BACILLUS AKTHRACIS IN RAW (FLAYED) GOAT SKIN AND LEATHER WITH REFERENCE TO PROCESSING METHODS

BY

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DIGITIZED

DECLARATION

I certify that this work was carried out in the Bacteriology Section of the Department of Pathology and Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. It was supervised by Professor P. B. Addo, Dr. A. A. Adesiyun and Dr. J. B. Adekeye.

The thesis is original and no part of it has been submitted elsewhere for a degree or diploma. Where use was made of other investigator's work, it was referenced and acknowledged.

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CERTIFICATION

This thesis entitled B. anthracis in raw (flayed) goat skin and leather with reference to processing methods meets the regulation governing degree of Master of Science, Ahmadu Bello University, Zaria, and it is approved for its contribution to scientific knowledge and literary presentation.

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ABSTRACT

A bacteriological survey of fresh flayed goat skins, locally tanned and scientifically tanned goat leather was conducted for Bacillus anthracis.

Eleven isolates of Bacillus anthracis were recovered from fresh-flayed skins. This represents 10.8 percent of the total number of raw goat skins examined. Of the one-hundred and three samples of locally-tanned leather samples examined, only 8 (7.8 percent) seemed contaminated by Bacillus anthracis while in the cases of scientifically-tanned leather samples examined, 7(6.8 percent) were contaminated by Bacillus anthracis. All virulent B. anthracis isolates killed mice within forty-eight hours post inoculation.

Bacillus anthracis survived tanning processes only up to bating stage. At the pickling stage, all bacteria were eliminated with no recovery of any organism at that point or beyond.

It is concluded that the micro-organisms including anthrax bacillus recovered from finished products are due to post production contamination.

The public health aspect of these findings are emphasized.

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Chapter One

INTRODUCTION

Anthrax is a disease of warm blooded animals particularly cattle, sheep, goats, buffaloes and also man. In domestic herbivores, including cattle, sheep and goats, the disease is usually an acute, febrile one with sudden onset and rapid death. In man, the infection may take the acute form but the cutaneous form is more common (Gibsons, 1963). The cutaneous form develops primarily at the site of a scratch or abrasion and the lesion is characterized by a small red macule, often pruritic, leading to an extensive oedema and this may result in necrosis. The spores of the organism are said to be carried on hides and skins of normal animals, especially cattle, sheep, goats and or some leather products (Jarolimek, 1960; Wilsons and Miles, 1964).

The aetiological agent of anthrax is Bacillus anthracis. It was discovered and isolated first by Rayer in the blood of sheep (Rayer, 1850; Davaine, 1863-1864; Koch, 1874).

Anthrax is of great economic importance and the incidence of this disease in Nigerian cattle, sheep and goats is of great concern to Nigerian leather-

scientists and technologists. This is partly because of its resulting degrading effects on their processed or tanned hides and skins which pose a threat to the economy of Nigeria as regards the Leather Industry.

In addition, it poses great hazards to human life and it is one of the most dangerous and very important zoonotic diseases.

Beaton (1939) and Campbell (1955-1956) reported cases of anthrax in cattle, sheep and goats in some parts of Northern and Eastern Nigeria. They speculated that the incidence definitely higher ... but carcasses of affected animals are either eaten unknowingly or buried in extremis.

Tanned leather are generally found to be contaminated by many yeasts, moulds, fungi, and bacteria. Of these, the most commonly prevalent are spore - forming organisms of the genus Bacillus. Some of them are harmful, highly resistant and commonly encountered in many of the beam-houses or tanneries (Prevot, 1956, 1960; Venkatesan, 1979). The hides and skins processor or tanner must therefore become well acquainted with such valuable information and scientific knowledge in order to produce high quality leathers that are free of infectious pathogens. Thereby, he can increase his exports by producing improved quality goods.

The public health hazard is highly considered for the tanner working at the risk of being infected in the tannery. He must prevent this, as much as possible and of course treat his tannery waste effluent which might have been contaminated by the viable Bacillus anthracis or its spores to minimize possible public health hazards.

Little or no work has been recorded on the prevalence of Bacillus anthracis in Nigerian raw hides and skins of cattle, sheep and goats.

Its survival through the local and modern tanneries in Nigeria are unknown. There are also reports from elsewhere in the world on its prevalence on some livestock finished products and or post-tanning contamination of their products (Anonymous, 1969, Davies and Harvey, 1972).

The objectives of this study therefore, are:-

1. To determine the prevalence of Bacillus anthracis in raw goat skin.
2. To compare the carriage rate of locally tanned leather with that of the modern scientifically-tanned leather.
3. To determine the survival of Bacillus anthracis through modern scientific tanning process.

Chapter Two

LITERATURE REVIEW

BACILLUS ANTHRACIS AND ITS PUBLIC HEALTH SIGNIFICANCE IN LEATHER INDUSTRY

Historical Background to the Disease Casued by *Bacillus anthracis*

The genus Bacillus consists of the largest and well known of the genera of bacteria. There are over forty eight known species. Most of these species are not pathogenic for animals or humans, although some, such as *Bacillus subtilis* can cause infection if given an opportunity, and *Bacillus cereus* can grow in food and may lead to food poisoning in humans. The pathogenic species is *Bacillus anthracis*, the causative organism of anthrax. Generally bacilli are commonly found as laboratory contaminants, very abundant in soil, and some are pathogens of insects. These include *Bacillus firmus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus coagulans*, *Bacillus pantothenticus*, *Bacillus alvei*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus laterosporus*, *Bacillus macerans*. *Bacillus sphaericus*, *Bacillus*

stearothermophilus and other Bacillus species (Wolf & Baker Group I & II) (Cowan and Steel, 1965).

The term "anthrax" is derived from the Greek word "anthrakos" meaning coal, and refers to the black eschar typical of cutaneous anthrax (Lamb, 1973). It is known in Hausa as "chiwon saifa" (Beaton, 1939).

Anthrax is a disease of all warm-blooded animals including man. It is infact one of the oldest diseases recorded and available records indicate that anthrax is an ancient disease that has plagued man and his domestic animals for thousands of years (The Holy Bible, Exodus Chapters 7:1-7Klemm and Klein, 1959). It is one of the interesting diseases that is encountered in domestic animals as described by Gibsons (1963). Human anthrax has been associated with consumption of meat of animals dead of the disease or when in contact with contaminated animals that is, agricultural anthrax, or their by-products like hair, wool, hides, that is industrial anthrax (Merchant and Parker, 1971).

Historically, Bacillus anthracis was discovered in the blood of sheep by Rayer in 1850. Davaine in 1863-1864 described transmission of the disease

to normal animals by inoculation of infected blood, the appearance of the organism in the blood of recipient and its rapid increase until death of host. Koch in 1877, reported the isolation and cultivation of the organism, its formation of spores, the production of the disease with pure cultures, and the recovery of the organism from the experimental infection (Hubbert, McCulloch and Schnurrenberger, 1975).

The isolation of attenuated strains and their effectiveness as vaccines were demonstrated by Pasteur in 1881. The disease is enzootic over much of the cultivated area of the world and major epizootics with associated human disease have occured for centuries (Wolfgang, 1968).

In the United States of America, anthrax appeared during early settlement, and outbreaks of a disease consistent with anthrax was reported in Kentucky in 1824 (Hanson, 1959). It has been quite prevalent in Southern Europe, Asia, Russia and in Africa especially in Equitorial Africa (Gibsons, 1963; Wilson and Miles, 1964).

Beaton (1939) and Campbell (1955-1956) reported some cases in Nigeria (Sokoto and Abakaliki) and speculated that the incidence was definitely higher

but carcasses of affected animals were either eaten unknowingly or buried in extremis.

General Characteristics of the Genus Bacillus

Members of the Genus Bacillus are large, aerobic, Gram-positive (though old cultures decolourise easily), spore-forming rods. They are catalase positive many are fermentative and most are motile except B. anthracis.

Bacillus species occur widely in nature being found in air, water and soil. They are among the most common laboratory contaminants and are usually ignored when recovered from clinical materials of non-anthrax infected animals. The various species of Bacillus produce a variety of colonies. With experience, one can frequently identify them by their characteristic morphology and texture. Some general features of colonies are the following:

B. cereus - Hemolytic, greenish-grey colour and ground glass appearance

B. mycoides - Mold-like or lobular-like colonies

B. mesentericus and other species produce slimy colonies that are sticky and mucoid.

B. subtilis - Many strains are beta-hemolytic and colonies are rough, opaque, and spreading; slimy colonies are encountered and yellow, orange, or brown pigmentation is seen.

Growth in broth is frequently characterised by the production of a surface pellicle, another feature of these organisms which may result in confusion is the gram variability of a number of species (Tables 2:1, 2:2 and 2:3).

Distribution and prevalence of Bacillus anthracis

Typical of the Genus Bacillus, Bacillus anthracis is found all over the world as it contaminates air, water, soil, animal feeds and human foods (Davies and Harvey, 1972). Although, anthrax is widespread, it is particularly prevalent in countries where no organised control of animal diseases exists. India, China, Liberia, Russia, Northern Africa, some parts of South America and Mexico have anthrax as a major livestock problem (Klemm and Klein, 1959), but the relative incidence vary in different countries ((Fig. 1), Steme, 1967).

Table 2:1 Characteristics that distinguish B. anthracis, smooth and rough, B. cereus, and B. megaterium (all cultures incubated at 35-37°C)

Tests	B. anthracis Smooth	B. anthracis Rough	B. cereus	B. megaterium
Motility in agar	-	-	+	+
Hemolysis on sheep's blood agar (24hr)	-	-	+	-
Litmus milk peptonization (72hr)	-	-	+	V
Salicin fermentation	-	-	+	-
Growth on penicillin agar	-	-	+	-
Growth on bicarbonate (0.7%) agar in air in a candle jar	R S	R R	R R	NG SY
Death of mice within 24-48hr	+	-	-	-
Spleen smear, capsular stain of bacteria with polychrome methylene blue	+	-	-	-

V, variable, some strains positive, others negative; R, rough, dull, gray, flat colonies; S, smooth, mucoid, glistening, raised colonies, SY, smooth, yellow, dull, raised colonies; NG no growth

Source: George (1978)

Table 2:2 Sugar reactions for Bacillus species in medium with ammonium salt base

	B. anthracis	B. cereus	B. badius	B. firmus	B. lentus	B. licheniformis	B. megaterium	B. pumilus	B. subtilis	B. coagulans	B. vulvifaciens	B. pantothenticus	B. alvei	B. brevis	B. circulans	B. laterosporus	B. macerans	B. polymyxa	B. stearothermophilus	B. sphaericus
Gram reaction	+	+	+	+	+	+	+	+	+	+	+	+	v	v	v	v	v	v	v	v
Morphological group	1	1	1	1	1	1	1	1	1	1cr2	2	3	2	2	2	2	2	2	2	3
Motility	-	+**	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 65°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Anaerobic growth in glucose broth	+	+	-	-	-	+*	-	-	-	+	+	-	+*	-	d	+	+*	+*	+	-
Citrate utilization	+	+	-	-	-	+	+	+	+	-	-	d	-	d	-	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	-	+	d	d
Starch hydrolysis	+	+	-	+	+	+	+	-	+	+	-	+	+	-	+	-	+	+	-	-
Glucose (acid)	+	+	-	-	-	+	+	+	+	+	NG	+	+	+	+	+	+	+	+	-
Arabinose (acid)	-	-	-	-	-	+	+	+	+	d	NG	d	-	-	+	-	+	+	d	-
Mannitol (acid)	-	-	-	-	-	+	+	+	+	d	NG	-	-	+	d	+	+	+	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	d	-	-	-	-
V-P	+	+	-	-	-	+	-	+	+	d	-	-	+	-	-	-	-	+	-	-
Nitrate reduction	+	+	-	+	-	+	-	-	+	d	+	d	-	d	d	+	+	+	+	-
Urease	-	d	-	-	+	d	d	-	d	-	-	-	-	-	-	-	-	-	d	d
LV	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	d	-

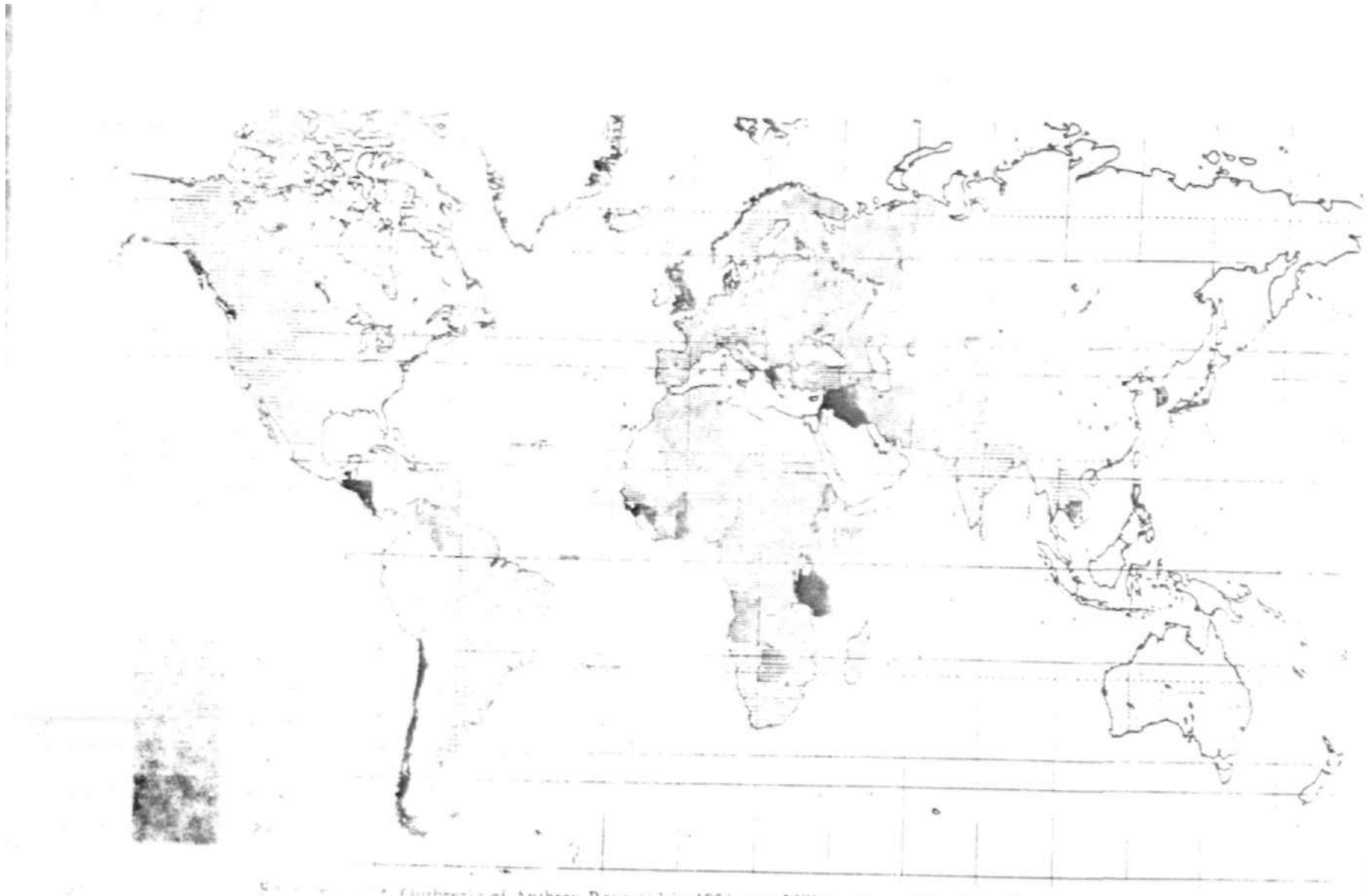
** B. cereus v. mycoides and non-rhizoid variants are usually non-motile
* Gas may be produced; NG no growth on ammonium salt basal medium v variable generally positive in young cultures.
SOURCE: Cowan and Steel (1965)

TABLE 2:3 - The identification of unusual pathogenic Gram-negative bacteria

Number of Strains	40 Bacillus cereus & mycoides*	10 Bacillus anthracis	7 Bacillus megaterium	20 Bacillus subtilis	2 Bacillus licheniformis	2 Bacillus firmus	16 Bacillus pumilus	1 Bacillus alvei	1 Bacillus stearothermopholis	50 Mycobacterium rhodochrous (Group)	II & ATCC Str. Brevibacter acetylicu
Gas from Glucose	-	-	-	-	-	-	-	-	-	-	-
Action on Blood	beta	- or slal	beta(ly)	beta(ly)	beta	- or LG	beta(-)	ly		-	- to al
Carbohydrates:Base Used	Ferm	Ferm	Ferm	Ferm	Ferm	Ferm	Ferm	Ferm	Ferm	OF	Ferm
Glucose	A	A	A	A	A	A	A	A	A	A	A
Xylose	-	-	-(lA)	-	-,A[v]	-	-	-	-	Alk-N	-
Mannitol	-	-	A	A	A	A	A	-	-	A(Alk)	A
Lactose	-(A)	-(A)	-.	-	-	-	-	-	A	Alk	-
Sucrose	A or -	A or -	A	A	A	A	A	A	A	A(N-Alk)	A
Maltose	A	A	A(-)	var	A	A	-	A	A	Alk(N-A)	A
Salicin	A	-(A,L)				-	-				
Arabinose	-	-				-	-(A)				
Starch hydrol				+	+		-	+,w			
Catalase	+	+	+(1-)	+	+	+	+	+		+	+
Oxidase	+(-)	+(-)	var	var	+,w+	-(Kovac +)	-(w+)	+		-(w+)	+(w or -
Growth Nutrient Broth	+	+	+	+	+	-	+	+	+	+(-)	+(-)
Growth MacConkey	-	-	-	-	-	-	-	-	-	-	-
Growth SS	-	-	-	-	-	-	-	-	-	-	-
Growth Simmons Citrate(Alk)	-	-	-	- or +	+	-	-(+[3-7])	-	-	-(+)	-
Urea-Christensens	-(+)	-(+)	var	+ or -	-,+[r]	-	-	+[r]	+,L	var	-
Nitrate Reduction	+	+	var	+(-)	+	+(NO_2+)	-	-	+	+ or -	-
Indol	-	-	-	-	-	-	-	+	-	-	-
Slant/Butt	A(Alk)/A	A(Alk)/A	A(Alk)/A	Alk(A)/A	A/A	A/A	Alk/A	A/A	A/A	Alk/N	A/A
H_2S-Butt/Paper	-/+ or -	-/+(-)	-/+	-/var	-/+,-	-/+	-/+(-)	-/+	-/+	-/+	-/
M-R	+(-)	var	- or +		-,+	-	var	+	+		-(w+)
V-P	-(+)/+[2]	-(+)/+[2]	-/-	+(-)/+	+/	-/	var/+	+/	-/		+(-)/
Gelatin Hydrolysis	+ rapid	-(wk)	+	+	+	+[7-14]	+	+		-	+
Litmus Milk	Pep or NC	Pep or NC	var	Pep	Pep,clt	-	-(Pep)	clt	A,w	Alk	Pep
Flagella(motile)	+(-)	NM	+	+	+	+	+	+	+	-	peri
Pigment			-	-	-	-	-		-	pink,coral,yellow	yellow
Gas,Anaerobic nitrate				-	+						
Cell Diam	≥ 0.9μ	≥ 0.9μ	≥ 0.9μ	<0.9μ	< 0.9μ	< 0.9μ	< 0.9μ	< 0.9μ	< 0.9μ		
Methylene Blue Red	+	-(L)									
Glucose NA ≥ NA,growth				+	+	-					
Growth at:25°;37°;42°C	+;+;+(-)	+;+;+(-)	+;+;+	+;+;+	+;+;+	+;+;+	+;+;+	+;+;+	-;-;+,(65°)	+(-);+;var	+;+;var
Esculin Hydrolysis	+(-)	-(+)?	+(2-)	+	+	-	+		+	-	+(-)
Fat granules	+	+	+	-	-	-					
Spores	Oval, do not swell cell	Oval, do not swell cell	Oval, do not swell cell	Oval, do not swell cell	Oval, do not swell cell	Oval, do not swell cell	Oval, do not swell cell	Oval, do not swell cell	Oval, do not swell cell; parallel in rows	Oval, small cell	-
Lechithinase	+ Wide Zone	negative or narrow zone									

Footnote: * rhizoid growth

Source: Elizabeth O. King: Preliminary Revision by Robert E. Weaver et al. July (1980) Pg. 12. CDC Atlanta, Georgia, U.S.A.



Outbreaks of Anthrax Reported in 1964 per Million Susceptible Animals

Source: Hubert et al. (1975)

Improved control of animal anthrax and a resulting decrease in human agriculturally-related anthrax have been noted in the United States for several decades. During the period of 1927 to 1939, the incidence of recognized outbreaks of animal anthrax increased in some countries, and decreased in others (Minnet, 1952). No adequate estimates of incidence are available from the areas in which the disease appears to be most prevalent. In many underdeveloped areas, available evidence indicates that anthrax has not been controlled and the incidence is increasing (Hubbert et al. 1975).

Outbreaks of animal anthrax cause serious losses for individual livestock farmers, and major epizootics with embargoes on shipment of animals and milk can result in significant losses for many farmers and reduction in foreign exchange earning to the countries involved. However, in the United States and in Northern Europe, the incidence of anthrax is low, and the overall impact of the disease on the nutritional status and economic condition of the population is minimal (Pienaar and De, 1967).

In undeveloped areas with higher incidence of the disease, and in which population exists near the starvation level, the loss of critical animal protein

caused by anthrax must reduce significantly the adequacy of the diet. Concern has been expressed that epizootics in wild animals when added to the pressures exerted by encroaching civilization, may contribute to the extinction of species (Heyworth, Voos, Ropp, Meinel and Darlow, 1975).

Beaton (1939) reported that epizootics of anthrax in goats are stated by goat owners to occur in certain swampy areas of Sokoto Province of Nigeria and individual cases have been recorded where the cause of death was definitely established. Also, Campbell (1956-1957) reported two outbreaks in Abakaliki division and three other outbreaks in the former Eastern Region of Nigeria. There is no doubt the incidence of the disease is higher than reported, since about 10 percent of Nigerian hides exported to Britain are presumed to possess anthrax spores (Campbell, 1955).

The low incidence rate as reported is probably due to the fact that such anthrax infected animals are killed in subacute or chronic cases, and even when they die in the per acute cases, the carcasses may be eaten: or buried without reporting to the Veterinary authority. The enzootic nature of the disease with only few animals being involved and

the poor disease reporting system generally in the country may also account for the apparently low reported cases of anthrax in livestock in general. In human anthrax, infection takes the cutaneous or pneumonic forms with non-characteristic lesions or symptoms. The disease in humans could therefore be mistaken for other infections.

Growth requirements and characteristics of Bacillus anthracis

Bacillus anthracis grows readily on ordinary bacteriological media such as blood agar, bicarbonate agar and nutrient agar, under aerobic conditions. It is isolated easily from the blood or internal organs of an infected animal.

The optimum temperature for growth is 37°C and a slightly alkaline medium with pH 7.5-7.8 is most condusive for good growth.

On either blood agar or nutrient agar surface, the colonies appear dull, opaque, greyish-white with an irregular border from which long strands of cells are seen in parallel "medusa-head" or 'curled-hair' or 'barriesters-wig' appearance.

As the colony ages, "vesicles" may appear on the surface, giving it a contcured appearance. Broth is turbid with flocular growth on the surface which sinks to the bottom of the tube within 24 hours.

In a gelatin stab culture, filaments of growth radiate from the line of puncture and give rise to the appearance of an inverted fir tree (Lamb, 1973).

Aging and other treatment of stock laboratory cultures produce variants of different types.

To produce spores on the laboratory media, B. anthracis is incubated in 10-20% of carbon dioxide. Pasteur, was the first to note the loss of spore-formation by exposure to 42°C.

Chamberland and Roux (1959) observed that asporogeneous strains could be produced by contact with phenol, 1:800 and potassium dichromate 1:2,000. Bormans (1952) reported that a permanent asporogeneous type can be produced by growing the organism repeatedly in sheep, calf, horse or dog sera. The organism loses its capsule when grown on artificial laboratory media unless the medium is incubated in an atmosphere of 10-20 per cent carbon dioxide. This loss causes colony variation. Contrary to the

usual type of variation, the large rough strain or capsulated with curled edges is the normal and virulent whereas the smaller smooth strain is avirulent, typical of materials obtained directly from the tissues.(Merchant and Parker, 1971).

Biochemical reactions of B. anthracis

B. anthracis produces acid but no gas from glucose, sucrose, maltose fructose, trehalose, and salicin. There is no fermentation of lactose, galactose, arabinose, rhamnose, mannose, raffinose, inulin, mannitol, dulcitol, sorbitol, inositol, and adonitol. Indole and hydrogen sulphide are not produced; nitrates are reduced to nitrites; and from nitrites, ammonia (NH_3) is produced. Methylene blue is reduced by some of the more virulent strains, and some strains hemolyse sheep blood (Burdon, 1956).

B. anthracis is methyl-red positive, and Voges-Proskeur variable. Litmus milk is coagulated decolourized and slowly peptonized. It is not motile, pathogenic for laboratory animals, usually sensitive to about 10 units per ml penicillin and most strains are sensitive to a specific bacteriophage especially the Gamma-phage (Burdon, 1956).

Resistance and sensitivity of Bacillus anthracis

The vegetative cells of B. anthracis are not more resistant than similar cells of other bacteria. This means that the vegetative cells can be destroyed by ordinary laboratory disinfectants.

However, the spores formed by the organism are very resistant and the disinfection against the spore are not dependable (Solyts, 1963). Spores will remain viable for years and will be as virulent as the culture proved to be when originally isolated (Wilson and Rusell, 1964).

Pasteur (1935) showed that spores remain viable in buried carcass for twelve years. Gaiger (1959) also reported that spores could be germinated after 15-24 years duration in the soil. Instances have been reported where animals became infected on premises 25 years after the original cases of the disease. Hair, wool, bone meals, animal feeds, and hides not treated sufficiently to kill spores may remain infective for years. The viability of B. anthracis in the tissues of artificially infected guinea pigs exposed to decomposition, temperature and dessication, was recorded by Stein in 1947. The organism is rapidly destroyed by decomposition in

unopened carcasses after the third day, except under temperatures of 5°C - 10°C, when it survives at least for 4 weeks (Merchant and Parker (1971).

Repeated freezing at -72°C and thawing at 37.5°C may kill vegetative cells but probably not spores. Anthrax spore suspensions were viable for 90-124 days when kept at -60°C to 70°C and for nine to 10 years at either -3°C to -6°C or at 5-10°C. Blood swabs from field cases of anthrax kept in the light at room temperature were still virulent up to 9 years. Also agar slant cultures of B. anthracis isolated from a field case in 1928, kept sealed with paraffin and stored in the dark at room temperature was f und to be virulent at the end of 38 years (Merchant and Parker, 1971).

In testing the resistance of anthrax - spores to heat, Stein & Rogers (1945), used 43 strains. All of these strains were killed by vigorous boiling within 3-15 minutes. Spores from 31 strains were killed in 5-15 minutes at 100°C to 101°C in the steam sterilizer, but at 90°C - 91°C only 6 out of 12 strains were destroyed in 60 minutes. In the autoclave, at 120°C all of the 31 strains tested were destroyed in 5-15 minutes. Insistence that cultures of B. anthracis must not be sterilized in

an open vessels or glass tube is supported by the work of Stein and Rogers in 1946, who found that viable spores escaped with steam from such cultures.

Solyts (1963) observed that spores of the organism in film on slides may withstand the temperature of the bunsen flame for 5-6 seconds, that none of the common staining methods destroy all spores, that mercuric chloride 1:1000, killed the spores on slides in 5 minutes and did not alter the smear, whereas a saturated solution of potassium permanganate ruined the smear for observation purposes. In view of these results, it appears prudent to treat smears of B. anthracis with mercuric chloride 1:1000, for 5 minutes and then one can proceed with desired staining technique.

Ten per cent formalin at 40°C, will kill anthrax spores in 15 minutes, but when this chemical is used at lower temperatures, more time must be allowed for disinfection. Freshly prepared NaOH in a 5% solution is a satisfactory agent for disinfecting objects contained by the spores of B. anthracis (Minnet, 1952; Merchant and Parker, 1971).

Antigenic structures:- Toxins and phage-lysis of B. anthracis

There appears to be two antigenic substances in B. anthracis One protein like material is found in the capsule and the other is a somatic polysaccharide antigen. Both antigens act specifically with precipitating sera.

Lamanna and Eisler (1960) described the antigens specific for the endospore phase of B. anthracis and B. cereus but were unable to distinguish between the two organisms by agglutination tests.

B. anthracis produces two toxic substances when grown in a casamino acid medium without serum or other protein (Thorne et al., 1960). One component called protective antigen, was present in the filtrate after passing through filtered glass filters and the other called filter factor was adsorbed to the filters. When tested alone, each factor was non-toxic, but when used together skins reactions were noted in guinea pigs and they were lethal to mice.

Stanley and Smith (1961) have found three factors in anthrax toxin. Factor I, a proteinaceous material with a strong chelating material for metal ions was

not toxic when given alone, but when mixed with purified factor II which is the protective factor for anthrax, oedema was produced in the skin of rabbit, while mice were killed. Factor III which is a rat lethal factor was different antigenically from factors I and II. It was present in anthrax toxin produce in vivo. It can also be produced in vitro only under special conditions. It was non-lethal to mice when injected alone but lethal when mixed with Factor II not Factor I. All these three factors were found to be more virulent when mixed together than any other combinations.

Cromartie (1947), in a series of studies gave significant information which has helped in understanding B. anthracis disease. Two substances are present in crude extracts of lesions produced by the organism, one an inflammatory factor capable of damaging tissue and the other, a protective antigen responsible for its immunizing property.

The tissue-damaging factor is capable of interfering with the blood clotting mechanism and inhibits an anthracidal substance produced by leucocytes and other cells. The protective antigen migrates in an invisible component intermediate between the gamma and beta globulins. It is destroyed

by heating at 57°C for 30 minutes and also by trypsin but not destroyed by formalin in a final concentration of 1.0% and is stable in lyophilised state. This antigen has not been purified, but fractionation with ethanol in the cold suggests a method of concentration.

Host susceptibility of Bacillus anthracis

Essentially, all mammals are susceptible to infection by B. anthracis. Infections of varying severity occur naturally or may be produced experimentally in a wide variety of hosts.

From the standpoint of production of disease in man, infection of domestic herbivorous animals is most important and disease in these animals is also responsible for the greatest economic loss. Cattle, sheep, horses and goats are infected most frequently, the relative incidence varying in different countries. Among wild herbivorous animals, the disease occurs most frequently in deer, antelope and buffalo (Bienaar and De, 1967). Carnivorous and omnivorous animals, both domestic and wild, are infected by feeding on infected carcasses. Dogs, swine, mink and a variety of animals in zoos have been infected in this way Stiles, 1944;.(Stein and Manness, 1955; Lyon, 1973).

Among experimental animals, mice, guinea pigs, and rabbits are highly susceptible, while rats are susceptible at birth, but acquire a high degree of resistance early in life (Taylor, Kennedy and Bludell, 1962).

Monkeys and chimpazees are readily infected experimentally. Albrink, (1959) reported that cattle are relatively resistant to subcutaneous challenge but fatal infection can be produced by feeding. Relative susceptibility by various routes of infection varies in different animals. Thus, fatal infection is readily produced in guinea pigs and rabbits by the subcutaneous route, although the animals are resistant to massive oral challenge.

Route of entry into host and pathogenicity of *Bacillus anthracis*

In animals, infection is usually through alimentary tract after ingestion of infected food, but direct infection from animal to animal is rare. Among herbivores, the disease is usually fatal appearing as septicemia in cattle, sheep, horses and mules in an apopletic form, an acute form and in a sub-acute form, and in these death occurs rapidly. Just before death, the animals excrete the bacilli in the urine, faeces, and saliva,

contaminating the surrounding with the spores of B. anthracis (Calvin, 1969).

Cutaneous anthrax in animals is uncommon but has been described (Lamb, 1973). It is usually acquired from biting flies which carry the spores passively. Infection by inhalation is not thought to occur naturally in animals but it has been shown experimentally (Knight, Wyne-Williams and Willis, 1969).

The type of anthrax in man usually depends on the avenue of infection. Pulmonary anthrax, or wool-sorter's disease is contracted by the inhalation of anthrax spores. The disease runs its course as a typical pneumonia and is usually fatal. Cutaneous anthrax or malignant carbuncles result from skin infection through scratches or other breaks in the skin. The pustules start as a small red pimple and gradually spreads forming a large swelling with a dark centre surrounded by small vesicles (Taylor and Carslaw, 1967; Merchantand Parker, 1971).

If the organism (B. anthracis) enters the blood stream, septicemia results. Most cases of cutaneous anthrax recover (Sen and Minett, 1956; Stein, 1963).

Nalin (1967) reported that intestinal anthrax results from the eating of uncooked meats from an anthrax carcass and it is extremely rare in civilized

countries. Cases of anthrax meningitis in man have also been reported (Al-Dulaimy and al-a laf, 1978).

It has been generally assumed that death of an animal or man following a rapid septicemia course of anthrax is due to the changes produced in vital tissues such as spleen, liver, kidneys, and heart muscle. Futhermore, it has been postulated that there are so many anthrax bacilli in the blood vascular system that they, because of their aerobic nature, utilize so much of the oxygen that the subject dies of asphyxia (Klein, 1963).

Clinical findings in anthrax

Clinically, in cattle, sheep, horses, goats and mules, massive hemorrhagic swellings may be present in the subacute type but are not so common in the apopletic and acute types. In swine, the disease is manifested by an acute pharyngitis with extensive oedema in the region. Anthrax in cats, though rare, is similar to that in dogs with the exception of a severe gastro-enteritis.

On post-mortem examination, anthrax is characterised by oedematous infiltrations in the sub-acute; hemorrhages in various parts of the body,

especially in nervous membranes, marked swelling of the spleen which is dark red and soft (respberry jam consistency) termed as splenic fever is observed.

Hemmorhages are often observed from the natural body openings with blood oozing out and remaining unclotted.

In man, the pulmonary anthrax runs its course as an atypical pneumonia. In cutaneous forms there are pustules with dark centre surrounded by small vesicles and regional lymph nodes are swollen and painful.

Laboratory diagnosis of anthrax

The laboratory diagnosis of anthrax in man and animals depends upon isolation and identification of Bacillus anthracis.

Smith, 1973, regarded B. anthracis as a pathogenic variety of B. cereus but Leise et al. (1959), and Burdon and Wende (1960) retained B. anthracis as a separate species. Among the features of B. anthracis used by Leise et al. (1959) to distinguish it from B. cereus were

1. Lysis by specific (gamma) bacteriophage
2. It's failure to produce hemolysis on blood agar. (B. cereus is hemolytic on sheep blood).

3. The production of mucoid colonies by virulent strains growing on bicarbonate agar in an atmosphere of carbon dioxide (avirulent strains and those of B. cereus do not do so).

But for the isolation and identification of B. anthracis the following steps of specimen collection and laboratory procedures have to be followed.

(A) Specimen collection

All animals suspected of having anthrax should be handled only by a veterinarian, and post-mortem examination should be done with extreme caution to prevent infection of people and contamination of the soil and environment.

Specimens for the laboratory should be collected as soon after death as possible, usually, specimens collected from decomposed carcass are of little value because B. anthracis may be destroyed or over-grown by putrefactive micro-organisms. If animals have been treated with antibiotics prior to examination, anthrax bacteria may be impossible to isolate from specimens (Biegeleisen et al., 1962). Specimens

for laboratory examinations may include the following:-

1. Smears from blood or spleen made on clean glass slides.
2. Sterile cotton swabs, gauze, or suture tape impregnated with splenic pulp and then placed in sterile sealed containers; and
3. a few milliliters of blood drawn especially with a sterile syringe and placed in a sterile sealed tube.

Rapid submissions of specimens to the laboratory is most important since B. anthracis can be readily isolated from fresh materials that are not grossly contaminated with other micro-organisms. In cases of suspected anthrax in which the signs and pathology are not characteristic, samples are best taken from localised oedematous areas or from lymph nodes, particularly those in the cervical region.

In typical cases of anthrax, a presumptive diagnosis may sometimes be made by microscopic examination of stained smears from the blood or spleen, particularly if the specimen is fresh.

However, it must be emphasized that this diagnosis is presumptive, since many other bacilli microscopically resemble B. anthracis. Ordinary stains do not demonstrate the typical appearance of virulent anthrax bacteria when they are present in fresh specimens.

Giemsa or Wright's stains can be used, but the simplest effective method is staining with polychrome methylene blue. When this is used, virulent encapsulated cells of B. anthracis appear as short chains of blue rods with square-ends, surrounded by pink amorphous material that probably represents, remnants of the capsule (McFadyean, 1903).

Methods in which fluorescent - antibody are used to identify B. anthracis are not specific and cannot yet be used for diagnosis. Tissues from spleen or lymph nodes for histologic examinations are not essential because the aetiologic diagnosis of anthrax depends on isolation and identification of B. anthracis.

(B) Laboratory Procedures

(i) Cultural diagnosis

Fresh specimens collected from uncontaminated source onto cotton swabs, gauze or suture tapes are wetted with a few milliliters of sterile nutrient broth and shaken for a few seconds and then the broth is streaked with an inoculating loop on plates of blood agar and bicarbonate agar. Samples of the blood can be streaked directly on the same two media.

If specimens are heavily contaminated they may be heated to 63°C for 5 minutes before streaking. This is to eliminate many of the non-sporing contaminants, but in order to avoid destruction of vegetative anthrax cells that have not sporulated, the original sample should also be streaked before heating.

Anthrax bacteria are difficult to isolate from sources such as bone meal, animal feeds, hair, hides and wool that may be heavily contaminated with other micro-organisms. An effective procedure is to cut about 10-20g of the specimen into small pieces, place the pieces in a sterile container, cover them with two percent aqueaous phenol and

allow the mixture to stand with occasional shaking for 30 minutes. The material is then re-suspended before the suspension is streaked directly on blood agar and bicarbonate agar. This method enhances the likelihood of recovering B. anthrax, because anthrax spores are quite resistant to phenol. Samples can also be heated as previously described, or they can be soaked in caustic agents such as potassium hydroxide (KCH) for about twenty to thirty minutes before streaking.

Selective media that permit growth of B. anthracis but inhibit growth of various other micro-organisms have not been widely used (Morris, 1955).

Cultures are incubated at temperatures between 25°C and 37°C. Most important is incubation of bicarbonate agar plates both aerobically and in candle jars.

Colonies will appear on agar plates within 24 hours of incubation at 35°C - 37°C and characteristic colonies of rough anthrax could be seen.

An important colony characteristic of virulent anthrax bacilli is the appearance on bicarbonate agar when the plates are incubated in the atmosphere

of carbon dioxide provided by a candle jar or anaerobic culturing jar. Under these conditions, virulent B. anthracis may be readily distinguished from avirulent strains and from B. cereus and B. megaterium. Colonies of virulent B. anthracis are unique, being smooth, grey, mucoid, and glistering with a brightly reflecting surface.

In contrast, the colonies of avirulent anthrax strains or of B. cereus are rough and show a dull surface. If bicarbonate agar plates are incubated in air, colonies of virulent and avirulent B. anthracis, B. cereus and B. megaterium all appear rough, with a dull, grey, ground-glass, surface that neither glisters nor reflects light.

(ii) Differential reactions from other species of Bacillus

Differentiation of B. anthracis from B. cereus or B. megaterium causes the most confusion. The three species can be easily differentiated by different tests as shown in Table 2:1.

Leise et al. (1959) described the presence or absence of growth on agar containing penicillin as an important differentiating criterion since virulent strain of B. anthracis are sensitive to penicillin.

This sensitivity is probably responsible for the 'String of Pearls' phenomeneon. But no one characteristic will differentiate strains of B. anthracis from other members of the Genus. For instance, although there have been no reports of motile strains of B. anthracis, non motile strains of other species such as B. cereus or B. megaterium were found. Similarly, some strains of B. cereus are sensitive to penicillin. In particular, the rough strains of B. anthracis may resemble B. cereus. Some rough strains are resistant to penicillin, others produce hemolysis while others effect peptonization of milk. The colony morphology on agar media, including bicarbonate agar incubated under carbon dioxide is similar to that of B. cereus.

However, a single rough strain will not exhibit all the characteristics of B. cereus and thus can be identified as a strain of B. anthracis.

(iii) Animal inoculation

Mice or guinea pigs are preferred for laboratory inoculation. Mature hash mice of 15-20g are as useful as purebred types.

Typical colonies grown on either blood agar or bicarbonate agar are emulsified in a few milliliters of saline. Mice are inoculated subcutaneously in the thigh or abdominal region with 0.1ml or with 0.5ml intraperitoneally. Injection of virulent B. anthracis will cause death within 16-48 hours. If injected mice are not dead at the end of 72 hours, the suspected organism is probably an avirulent B. anthracis. Death before 16 hours is also not usually due to anthrax (George, 1978).

All dead animals are autopsied. Smears are made from the spleen and blood and heart blood is inoculated onto bicarbonate agar. Autopsies, are carried out with caution, preferably in dissecting trays. Liberal use of disinfectants both on the carcass before it is opened and on the dissecting tray itself is advisable. A wise procedure is to place the animal's carcass on paper towels soaked with a disinfectant such as 1:1000 mercuric chloride or 5 per cent phenol.

Animal inoculations are best carried out with suspensions of pure cultures, because contaminating organisms can limit the in vivo growth of B. anthracis, or cause death of the injected animal earlier than anthrax infection. However, if need arises,

contaminated material or impure cultures can be inoculated into mice or guinea pigs by the scratch method, in which a few drops of material are rubbed into a previously scarified area of skin. Anthrax bacteria can usually penetrate the skin and establish an infection more readily than contaminating organisms. When this procedure is employed, the laboratory must be certain that virulent forms of B. anthracis are isolated and identified from injected animals that die.

Animal pathogenicity must be demonstrated before identifying a Bacillus as a virulent strain of B. anthracis. An important sequence to animal pathogenicity is recovery of typical virulent anthrax bacilli from heart blood and demonstration of capsules in vivo. This is done by using smears from the cut surface of the spleen and stained with polychrome methylene blue.

(iv) Immunological and serological diagnosis

Although the species of Bacillus have a number of antigens, serologic procedures are not of value for differentiation. A complex group of toxic materials are produced by B. anthracis but they are not used for identification.

The ascoli-thermo-precipitin test used for presumptive post-mortem diagnosis of anthrax depends upon detection of heat-stable antigens in tissues. The tissue is ground in saline and heated at 100°C and the filtered extract is layered over appropriate anthrax antiserum. A precipitate forms at the interface, if reactive. The antigens responsible for the reaction are shared with other bacilli, which are essentially saprophytes and are not likely to proliferate extensively in animal tissues and the test may be of value when cultural methods of diagnosis are unsuccessful.(Buchanam et al., 1975).

Because of the acute course of the disease, no specific antibody response is useful for diagnosis of present infection, and until recently, serologic methods were not sufficiently specific or sensitive to be of significant value in retrospective diagnosis or epidemiologic studies. The major capsular and somatic antigens of the organism are shared with some other bacilli, and the chain formation and spontaneous clumping of suspensions of the organisms make agglutination titrations impractical. Recent studies, with protective antigen, the major antigens in acquired immunity to infection have changed this picture. Available evidence indicates that protective antigen is restricted to Bacillus anthracis.

(Angelety and Wright, 1971). Sensitive and specific methods have been developed for determination of antibodies reactive with it. These include inhibition of line-formation of in double-diffusion precipitating reactions (Ray and Kachell, 1964), passive-haemagglutination reactions and antigen combining power tests.

(v) Phage-typing of Bacillus anthracis

Specific bacteriophages are available for B. anthracis but as Wright (1954) points out, no one strain of bacteriophage will lyse only non-encapsulated strains. For identification, the gamma or W phages are used since these phages are apparently most specific (Leise et al., 1959).

However, none of the phages are as yet readily available, and for this reason, susceptibility to phage is not included in the procedure for identifying virulent strains of B. anthracis. Lysis by bacteriophage, is rather a confirmatory test that can be carried out in special laboratories.

A visualised problem is an anthrax disease that arises from infections with vaccine strains of B. anthracis. Because these strains are usually rough, they do not form smooth colonies on bicarbonate agar medium incubated in a candle jar or anaerobic jar, nor are they pathogenic for mice within 24-48 hours. However, these strains can usually be differentiated from B. cereus by other biochemical reactions. The question of whether a vaccine strain caused anthrax in an animal must then be decided by the veterinarian once a culture has been identified as a rough strain of B. anthracis.

Immunity as regards Bacillus anthracis infection

Immunity is a lack of susceptibility on the part of a host as regards diseases with B. anthracis. Natural immunity or resistance of certain animal species is very high. This is a consequence, at least in part of an anthracidal activity present in tissues and associated with a histine-like protein or polypeptide.

Anthrax was one of the first disease a definite immunity was produced by the use of bacteria. Recovery from infection in the susceptible animal results in a solid immunity, and the serum

contains protective antibody to high titer. Nevertheless, it has not been possible to produce any appreciable immunity to challenge with killed vaccines of anthrax bacilli. An effective immunity has resulted only from infection with attenuated strains, as used by Pasteur in his early studies on immunity in anthrax in sheep which has a relatively resistance to anthrax or by inoculation with virulent strains in conjunction with protective antiserum. With examination of the immunological activity of the products in vivo growth of anthrax bacilli, in connection with the toxin, it was observed that, antigen eliciting an effective immune response was produce in vivo, but not ordinarily in culture in vitro (Cromartie, 1947: Merchant, Parker, 1971). In 1946, Gladstone found that the immunizing antigen could be produced in vitro in the presence of body fluids containing albumin and a dialyzable fraction replaceable by sodium carbonate. The elaboration of this antigen in culture and its nature have subsequently been studied at length (Strange and Betton, 1954).

The immunizing antigen is present in cell-free filtrates of *in vivo* or *in vitro* cultures, and apparently diffuses freely from the bacterial cells. It is heat-labile and somewhat unstable but can be preserved by lyophilization, and is proteinaceous in nature. It is formed in culture in chemically defined but complex mediums containing many amino acids, purines, glutamine, thiamine, bicarbonate, and other salts (Wright *et al.*, 1954). Certain of the amino acids, bicarbonate and calcium are required for formation of the antigen but not for growth. (Puziss and Wright, 1963). It may be precipitated by alum (Wright, 1954), or salted out and purified by adsorption and elution (Strange and Thorne, 1958). The activity is present in maximal amount in young cultures, and there is reason to believe that it tends to be destroyed by bacteria polypeptidases (Schlingman *et al.*, 1956).

This immunizing antigen is not related to the glutamyl polypeptide capsular substance, although both are required for virulence, and the antigen is produced for avirulent non-encapsulated mutants. It is intimately related to the toxin described above, but the relationship has not yet been precisely defined.

While an effective immunity is produced by prophylatic immunization with the immunizing antigen, in man as well as monkeys and other experimental animals, the nature of the immunity so produced is not completely clear (Ward,1965). It presumably includes antibody to the aggresin-like antiphagocytic activity and is antitoxic in that the skin lesion produced by intradermal inoculation in the rabbit is inhibited by immunization; this inhibition has been proposed as a measure of immunity to the infection (Belton and Henderson, 1956).

Prophylactic or active immunization of man to anthrax is not quantitatively significant under ordinary circumstances, but effective immunization of cattle, sheep and other domestic animals can be of very considerable importance.

Immunization by inoculation with attenuated, partially avirulent bacilli, Pasteur's prophylactic, has been widely used for the immunization of domestic animals, although infection, "vaccine anthrax", may occur (Auerbach and Wright, 1955).

An alternative method and one which has been used in areas where anthrax is prevalent is Sobernhim's method. It consists of the simultaneous

inoculation of virulent spore suspension and immune serum from recovered animals. Either method tends to perpetuate anthrax infection. The possibility that an effective immunity may be elicited by inoculation with sterile, cell-free preparations of the immunizing, or protective antigen described above is of considerable practical importance. While it is fully established that an effective immunity is so produced (Auerbach and Wright, 1955) the - efficacy of the antigen is difficult to evaluate. The intradermal reaction noted above and correlation of protection with complement - fixing antibody titer (McGann, Stearman and Wright, 1960) have been suggested but an immunity index has been advised by De Arman (1961), which appears to be more informative and correlates with antibody titrated by gel-diffusion (Klein et al., 1962; Klein, 1963; Ray and Kachelí, 1964)

On the basis of the immunity index, Klein et al. (1962) found that non-encapsulated avirulent mutant used as vaccine gave a 10-15 - fold increase in immunity, the protective antigen a 1000-fold increase, but immunization with protective antigen followed by inoculation with live vaccine gave as much as 10-fold increase over that of

uninoculated animals. Such immunization procedures have not yet been tested on a large scale under natural conditions.

Prevention and control of Bacillus anthracis infection

Elimination of anthrax in animals represents the ideal method of minimizing human contact with the disease. Control of human anthrax depends primarily on minimizing human contact with anthrax-infected animals and with animal products contaminated with B. anthracis and secondarily on immunization of persons whose duties neccesarily involve actual or potential contact with the organism.

Methods of approaching this objective vary greatly in different areas, depending on the magnitude of the problem and the source of infection. In colder areas in which B. anthracis does not become established, the disease is sporadic, and the source of infection is usually imported feed or feed supplements that originate in endemic areas. Bone meal, the most important of these, may be rendered safe by proper autoclaving during preparation. Although mass immunization would not be economically justified to prevent sporadic outbreaks

of this type, vaccination of all healthy animals in the herd may be indicated when outbreaks occur. Vaccination will usually stop an outbreak in cattle within a few days (Sterne, 1959). Prompt diagnosis, isolation and treatment of sick animals and proper disposal of carcasses by complete cremation or deep burial are universally recommended. Necropsies should not be performed on animals dead of anthrax, to avoid hazard to personnel and further contamination of the environment. Carcasses of animals dying under conditions compatible with the disease must not be opened until anthrax has been excluded by microscopic examination of blood smears. Farmers must remain alert for the possibility of anthrax and understand the procedures for safe disposal of contaminated carcasses (Calvin, 1969).

When anthrax appears, quanrantine of the entire herd until two weeks after the last case is diagnosed is usually considered advisable. In dairy herd, the animals must be kept under close surveillance and any animal showing fever or other evidence of illness must be isolated and its milk destroyed (WHO, 1967).

A different situation exists in permanent enzootic areas in which spores are distributed in the environment. Under these conditions, widespread

immunization of susceptible animals represents the only effective approach. Non-encapsulated spore vaccines consisting of stable mutants of B. anthracis that have lost the ability to produce the polypeptide capsule, have largely replaced attenuated spore vaccines for immunization of animals. The vaccines are effective, well tolerated and readily produced in quantity (Sterne, 1959).

Experience in South Africa indicates that mass immunization repeated yearly for several years can virtually eliminate anthrax from enzootic areas, despite continuance of primitive agricultural methods. The extent to which effective control of anthrax in domestic animals will result in elimination of the organisms from the environment presumably depends upon such factors as the climate, the soil, and the significance of wild animals as reservoirs.

Control of the disease in wild animals presents a more difficult problem, since ordinary methods immunization are impractical. Attempts to control epizootics by disposal of contaminated carcasses, although probably helpful in the long term, have little immediate effect. In general, detailed study has not been directed to control of B. anthracis as it exists in the environment. Addition of quaternary

ammonium compounds to contaminated water holes apparently reduced the incidence of anthrax during an epizootic in wild animals (Pienaar and De, 1967), and further investigations of control methods of this sought would be desirable.

Until anthrax is brought under control in enzootic areas, there will be a significant incidence of contamination of animal products originating in such areas, and anthrax will continue to occur among workers handling the contaminated materials. (Sulkin et al., 1963). Economically, feasible methods of disinfection to render such products safe for handling and subsequent use is difficult because of their relative perishable nature and the great resistance of anthrax spores to chemical disinfectants and heat. However, a process for disinfection of hair and wool involving washing and treatment with warm formaldehyde solution has been in use in Great Britain for many years (WHO, 1967). All hair and wool entering that country from endemic areas is processed and industrial anthrax has been effectively controlled. There is no effective and economically feasible method for disinfection of hides and skins as recorded by Wolff and Heimann (1951). Treatment with formaldehyde vapour is effective for decontamination of enclosed areas such as textile

mills and laboratories (Young et al., 1970). Effective non-viable preparations, of protective antigen have become available in recent years of immunization of workers actually or potentially exposed to B. anthracis (Puziss and Wright, 1963). The preparations are well tolerated, and greatly reduce the incidence of anthrax in occupationally exposed textile workers (Brachman et al., 1962).

Immunization has been recommended for industrial workers, veterinarians and scientific personnel who may be exposed to infection (Gold, 1967). The antigen has been made available by the Centre for Disease Control of the United States Public Health Service in Atlanta, Georgia. A similar product is available in Great Britain through the Ministry of Health (Arion, 1965). In the USSR, non-encapsulated spore vaccines administered by the respiratory as well as by the cutaneous routes have been used extensively for human immunization; no significant untoward reactions were reported (Aleksandrov et al., 1961).

Despite the progress in control of anthrax in advanced countries, it is evident that much remains to be done to control the disease throughout the world. The problem can be attacked at various levels.

Perhaps the most urgent task is to apply established methods of immunization with non-encapsulated spore vaccine to control the disease in domestic animals in underdeveloped areas. Sterne (1959), has suggested that non-viable antigens can be developed that would yield immunity of greater duration than is now attainable with spore vaccines. Progress in this area would greatly facilitate worldwide control of anthrax by reducing the required frequency of immunization. Further understanding of factors determining survival and infectivity of the organism in the environment also could be of the greatest significance, because new concepts might evolve that would be applicable to control the disease in wild as well as in domestic animals. In the United States, and other industrialised countries, much remains to be done in applying established methods of industrial hygiene to prevent infection of workers and the general public. In substitution of plastics and synthetic fibers for natural materials has already altered the distribution of industrial anthrax and further reduction in the demand for potentially contaminated animal products may be anticipated. There is reason for concern, however, that the decline in the importance of the disease in advanced countries will steadily

reduce the impotus for attacking the problem on a world-wide basis.

Epidemiology of Anthrax

Spontaneous disease occurs in herbivorous animals that acquire the infection by ingestion of spores that probably enter the body through microscopic cuts, or abrasions of the oral or intestinal mucosa. When a pasture has been contaminated with anthrax spores, it may remain a source of infection for twenty to thirty years. Although it is impossible to determine the precise time of infection in a case of spontaneous anthrax, it is certain that the duration of the disease is only a few days. The infected animals remain asymptomatic until a few hours before death. Mortality in herbivorous animals is usually about eighty percent. Sporadic cases occured in United States in 1968 where 165 cases of anthrax occured in 35 farms in California. Many species of animals acquire the natural disease, and epizootics continue to occur in wildlife sanctuaries in Africa (Burrows, 1973).

The most common form of human anthrax is industrial anthrax, which results from contact with animal products, such as wool, hide, goat hair, skin and bones imported from Africa, the Middle East, and

Asia. Less commonly, anthrax is acquired in an agricultural setting from working with infected animals. One of the most recent episodes of anthrax in the United States resulted from imported bongo drums covered with animals hide containing the spores (Burrows, 1973). Cutaneous anthrax also has resulted from contact with finished products, such as sharing brushes made with animal bristles, ivory keys on a piano, and wool products (Burrows, 1973).

Treatment of B. anthracis infection

Prior to the development of the sulfonamides and antibiotics, anthrax was treated with only partial success by a combination of antiserum and arsenicals. While the number of cases is limited by the low incidence of the disease, it has been found that sulfonamides, are effective chemotherapeutic agents, and penicillin remarkably so.

B. anthracis is quite susceptible to penicillin and when used early in the course of the illness, is curative. The major difficulty is the lack of clinical suspicion of anthrax because of its rarity. Cutaneous anthrax may therefore be diagnosed as another illness, with prescription of an antibiotic

appropriate to that illness. With pulmonary anthrax, the diagnosis usually is made postmortem, as is the case with gastro-intestinal anthrax. If the diagnosis of pulmonary anthrax is made in sufficient time, large intravenous doses of penicillin should be instituted as quickly as possible.

In patients allergic to penicillin or in cases of penicillin-resistant strains, tetracycline may be used. If a skin lesion is mistakenly identified as a staphylococcal infection, incision and drainage may be attempted. However, this can lead to disastrous results because of widespread dissemination of the organism.

MICROBIOLOGY IN LEATHER MANUFACTURE

Processing of hides and skins to leather

The manufacture of leather whether in small scale at local level or by the industrial, commercial and modern techniques has some microbiological importance which is of great economic significance to the leather industry.

The chief processes used in leather manufacture (O'Flaherty, Roddy and Lollar, 1965), consist of the followings:-

A. Before tannage (in normal sequence)

i. Flaying:- removing the skin from the animal

ii. Curing:- to preserve skins during transport or storage.

iii. Washing:- (wet-salted material) or soaking back (dry material) - to restore "cured" hides and skins to a natural raw condition.

iv. Liming:- to loosen hair, fat, flesh, etc., and "plump" up the skin ready for tanning.

v. Unhairing:- to remove hair

vi. Fleshing:- to cut away unwanted fat and flesh.

vii. Deliming:- to neutralise the alkali from (iv).

viii. Bating:- drenching or scouring- to bring the skin to the right acidity for tannage. If the skins are pickled, they may be preserved at this stage and in some cases degreased.

B. Tannage

The hides and skins are now tanned by whichever method is appropriate, using vegetable tans or mineral (chrome or alum or oil) etc.

C. After Tannage

The order of processes varies very considerably for different leathers, the following is a general guide:-

i. Heavy leathers:- e.g. sole (or bottom) leathers

Washing or scouring - to remove surplus tans

Setting out - to make the grain flexible and of good colour

Stuffing - to impregnate with oil and fat for waterproof or mechanical leathers.

Drying

Rolling - to compress the leather and make it firm and flat.

ii. Light leathers, e.g. shoe upper leathers

Shaving or splitting - to achieve uniform thickness

Washing - to free from surplus chrome salts.

Neutralising - to adjust acidity

Dyeing - get the required colour

Flatliquoring - method of applying oil to achieve softness or "handle".

Setting out - to remove creases and surplus moisture

Drying

Staking - to flex and soften the skin.

Finishing or seasoning - to improve appearance

Glazing - high pressure polishing or

Plating - in a hydraulic press to give a smooth, flat surface or

Embossing in a press to produce fancy design.

The hide processor or the tanner must become well acquainted with such valuable information and scientific knowledge in order to produce a high quality leather and to increase exports by delivering imporved quality goods.

Biochemically, fresh hide or skin consists of water protein, fatty materials and some mineral salts, the protein being the most important for leather making. This protein may consist of many types with the important ones being collagen which on

tanning gives leather and keratin, which is the chief constituent of hair, wool, horn and the epidermal structures. The approximate composition of a freshly-flayed hide or skin is as follows.

Water	Protein	Fat	Mineral Salts	Other Substances (Pigments, etc.)
		Cattle Goats sheep		
60-70%	35%	2% 2-10% 5-30%	0.5%	0.5%

Structural or fibrous proteins			Non-structural or Globular protein	
Elastin (0.3%)	Collagen (85-90%)	Keratin (2%)	Albumen) Globulins) 1%	(Mucins 0.7%(Mucoids)
Yellow fibre woven in collagen fibres	This tans to give leather	Protein of hair & epidermis contain sulphur	Soluble, non-fibrous proteins, often removed in liming etc.	Mucous materials associated with fibres

Source:- O'Flaherty, Roddy and Lollar (1965).

All animal skins are made up of the above constituents but the figure for keratin will vary widely, depending on the amount of hair present, and the figure for fat will also vary. The division between albumns and mucins is debateble (Thorstensen, 1969).

The role of bacteria in leather industry

Bateria in raw hides and skins:- The raw hide or skin if not properly preserved is likely to undergo deterioration due to micro-organism. Prevot (1959) reported that the flora of tanning liquors and of vegetable-tanned leather is numerous consisting of yeasts of which harmful ones include Petasospora rhodanensis, P. strasburgensis, Pichia pseudopolymorpho, Candida guillermondii and C. humicola hydrolyse the tannis or by the formation of the sediments which bring down the tannis. The study of molds shows that the most aggresive ones are Aspergillus niger, Aspergillus flavus, Penicillium groupe, frequetans. Paecylomyces variati and Dermtophilus congolensis, while the bacteria involved both pathogenic ones such as Bacillus anthracis, Mycobacterium tuberculosis and non-pathogenic ones such as Bacillus cereus, Bacillus circulans, Bacillus megatherium Bacillus panthothenticus, Bacillus licheniformis, Micrococcus luteus, Sarcina citrea, Pseudomonas species Proteus vulgaris, Achromobacter and Flavobacterium species.

Microbial defects associated with leather processing

(i) Putrefaction.

The bacterial action on fresh hide or skin is proteolytic and this is done by the enzymes they secrete with favourable conditions of moisture, temperature, hydrogen-ion-concentration (pH) and other environmental factors. In sequence, protein is hydrolysed by proteases to peptones, peptides, dipeptides, amino acids and finally to ammonia. Anaerobic organisms of the genus Clostridium are found to be capable of hydrolysing collagen significantly. Aerobic bacteria initiate the putrefactive process and create a favourable medium in the tissues which encourages further putrefaction and degenerative changes by anaerobes. Bacteria might penetrate from both the flesh and grain side of the hide or skin, but the majority of them penetrate through the flesh side (Venkatesan, 1979). This action helps in the hair or wool-loosening effect of the hide or skin, notable species of bacteria responsible are those of Pseudomonas, Proteus and Achromobacter. The putrefactive damage of hides and skins brought about by the action of bacteria is a defect of unsuitable and inadequate curing of the hides and skins. The

degradation is most marked in the epidermis fibroblasts and muscle cells. The bacterial attack can first be noticed by a rapid putrid ammoniacal smell and also by the fact that if the hair is pulled it comes off the skin or hide very easily, this being due to the deterioration of the hair pulp and the epidermal cells lining the hair pulp. This is known as "hair slip" (Koppenhoeffer and Somer, 1939, Stuart, 1940).

Roddy (1964) rightly pointed out that the putrefaction damage of hide or skin ultimately brought about pinprick marks, hair or grain slips (rubbed-off surface resulting into blotchy finished leather) and holes in such leather. The degree of degradation of the skin by the micro-organisms can be estimated by assessing the amounts of ammonia, free amino and carboxyl groups released during putrefaction.

About thirteen species of bacteria belonging to different genera were found to be associated with fresh and dried raw hides and skins of living animals (Nopitsch, 1954) but only some of these are found to be potentially pathogenic. These include aerobic ones in the genera Streptococcus, Staphylococcus, Micrococcus, Mycobacterium anaerobic ones such as Clostridium perfringes, C. lentoputrescens

and facultative anaerobe; Proteus vulgaris, Staphylococcus species were considered as the normal skin inhabitants (Prey, 1935) and often responsible for secondary infections of the skin. Anon (1963) and Blank (1937), reported that African goat skins examined for anthrax revealed an incidence rate of 33%. The bacteria flora of a flayed hide or skin may vary appreciably from one place to the other depending upon the contaminating microflora from air, soil and water because Bacillus anthracis was isolated from goat hair and aerosols in goat hair mills (La-force et al., 1970).

(ii) Red Heat

The main aim of the raw hide processor or the tanner is to preserve the hide or skin collagen against microbial degradation and restore it to a fresh-hide form before processing into leather. But, the curing of hides and skins satisfactorily poses an important problem for the leather industry, and meticulous care has to be taken to produce good quality leather. This is because the commonly used curing agent is sodium chloride (NaCl). It is either used alone or in combination with an antiseptice, or a bacteriostat, or a bacteriocide which interfere with

the metabolic activity of the disastrous bacteria. But, the baterial flora in common salt (NaCl) changes appreciably if the salt has been used once for curing, particularly if such salt was collected from a hide or skin which was initially staled prior to curing. Micro-organisms isolated from used salt were as expected found to be highly salt resistant. Salt curing brings about the death of bacterial cells and removes partially the lobular proteins. Such proteins are undesirable as they encourage bacteria growth and 'red heat'. 'Red heat' is discolouration of the flesh side of the skin varying in intensity and shade. It has been found to be due to the pigment produced by halophilic chromogenic bacteria such as Sarcinia and Micrococcus species. (Martin et al., 1961; Javanovitis, 1927). The bacteria involved in this "red heat" are those in the genera Sarcinia and Micrococcus. The source of contamination has been traced to the common salt used for curing and also observed that marine salts obtained by solar evaporation were highly contaminated. Rock salt has been observed to be practically free from such contaminations by these bacteria (Martin et al., 1961).

Generally, bacteria associated vary in their salt tolerance. Sarcina, Micrococcus and Staphylococcus are significantly tolerant while others such as Bacillus and Rhodococcus are moderately tolerant.

But Bacillus has been found to be the only member able to digest the hide substance and probably responsible for the hair slip without doing any damage to the hide fibre (Anderson, 1945).

Besides being responsible for the spoilage of fresh and salted hides and skins, bacteria also cause some detrimental effects to hides and skins during tanning in the beam house.

(iii) Fungal stippen

This is a defect of curing caused by a fungus, otherwise known as 'salt stippen'. It is attributed to the rupture of the grain tissue of the hide during the initial curing process. The damaged tissue is subsequently invaded by secondary bacteria (O'Flaherty Roddy and Lollar, 1965).

(iv) Loose and pimpled grains

During soaking, some of the bacterial actions cause loose and pimpled grains in heavy leathers. In addition, they cause small pits in the grain and loss of substance with a thin, flabby and loosely woven empty fibre structure in the corium in both heavy and light leathers. These bacterial actions are influenced by the temperature and time of soaking in soak liquors.

During liming, chemicals (sulphide-lime-amines), micro-organism or their enzymes with or without added alkalines or buffer salt with a resultant pH 12-13 are normally used. McLaughlin, Rockwell and Blank (1927), observed that non-sporulating bacteria were killed by the fresh lime while the sporulating bacteria survived. But such sporulating organisms could not multiply in the lime liquors. Unhairing by liming was indepedent of any bacterial action. The bacteria associated with liming are those in the genera Micrococcus, Sarcina and Bacillus. Bacillus subtilis, variant vallens isolated from lime liquor by Nandy, Sen and Das (1976), produced an enzyme that possessed unhairing action.

Collett (1923-1926) found that some spores of certain bacilli survived passively in lime liquors. Also, proteolytic and fat-splitting enzymes played no important role in modern liming, because enzyme unhairing favours the bacterial growth (Cordon, Everett and Windus, 1961).

In pickling (pH 1-2), it is generally accepted that pickled pelts can be stored and preserved for a considerable period as the extreme acidic reaction of the pickle liquor inhibits the growth of bacteria. Cattle hides infected with Bacillus anthracis were rendered sterile by pickling in a solution containing 3 percent hydrochloric (HCl) acid and 10 percent sodium chloride (NaCl) (Procter, 1922).

The environment during bating (pH 7.5-9) operations is very condusive to bacterial growth because of the warm temperature, ideal hydrogen-ion-concentration (pH) and the presence of abundant proteins mainly in solid form and partly in solutions. The isolated, micro-organisms include species of the genera Sarcina, Staphylococcus, Pseudomonas, Flavobacterium, Serratia and Bacillus.

The pigment - producing ones, Pseudomonas and Flavobacterium were more predominant and there were cocci, non-pigmented bacilli of all types including the ubiquitous Bacillus mesenterious and other soil, water and air-borne organisms (Line, 1948).

(v) White spots

Tanning of pickled pelts in a beam house or tannery is either by vegetable tanning or mineral tanning. The extremely acidic reaction of the tanning liquors;

Chrome tanning pH 2-4

Vegetable tanning pH 3-5

Oil tannage pH 6,5-10

makes it unlikely that bacteria will survive with the exceptions of Leuconostoc mesenteroides, Leuconostoc dextranicum, Bacillus mesentericus fuscus and Alcaligenes viscosus which are capable of giving rise to "white spots" due to the slimy fermentation on the hides suspended for drying (Jarolimek, 1960). The origin of these organisms has been traced back to their presence in the raw stock itself and they survived the beam house or tannery processes to show up the defect on the finished leather as "white spots".

Generally, tanned leather are contaminated by some groups of micro-organisms of which fungi and bacteria are persistently inclusive. However, it is clear that the prevalent spore-forming organisms of the genus Bacillus, are highly harmful and commonly encountered in the beam houses and tanneries. As many investigators have directed their attention to saltless curing, greater care has to be taken to guard against these organisms especially Bacillus anthracis because of its public health importance and significance to leather industry workers and users of leather products (Venkatesan, 1979).

Chapter Three

MATERIALS AND METHODS

A. Field Survey for Bacillus anthracis

1. Survey of fresh flayed goat skin for Bacillus anthracis

Source and type of sample:

The 102 freshly flayed goats skins used for this investigation were collected from Samaru and Sabon-Gari abattoirs, located at two different points in Zaria. Thirty were collected from Samaru abattoir and the rest 72 from Sabon-Gari abattoir.

For each sample, as soon as the goat was killed and flayed, the whole piece of skin was taken in a clean plastic bucket and tightly covered with its lid. The samples were brought immediately to the laboratory for bacteriological examinations.

Sampling technique:

The sampling was done in an ultra-violet rayed room for sterility purposes. The skin was spread in a sterile tray and sample-cuttings were done for the bacteriological examinations of the skin.

A sterile piece of wood measuring five-centimetre in length and four centrimetre in breadth with an area of twenty square centimetres (5cm x 4cm = 20 sq. cm) was used to measure five pieces from the skin. The cuttings were done at random with sterile forceps and scissors using a pair of sterile hand gloves. A total area of a hundred square centimetres (100 sq. cm) of skin was removed for the bacteriological examination.

2. Survey of Locally-Tanned goat leather for Bacillus anthracis

Source and type of samples

For each piece of locally-tanned goat leather, a sterile sample bottle was used for collection. The samples were collected from four different shoe-makers at Samaru and another four at Sabon-Gari markets in Zaria town. After collection, the samples were brought immediately to the laboratory for the bacteriological procedures. A total number of 103 samples were collected.

3. Survey of modern or scientifically - tanned leather for Bacillus anthracis

Source and type of samples

Sterile sample bottles were used to collect the scientifically finished pieces of leather used for this investigation. The only source of collection was the Footwear and Leather Goods Section of Leather Research Institute of Nigeria, Zaria. One-hundred and three samples were collected and brought to the laboratory immediately for the bacteriological examinations.

Isolation and laboratory procedures

Laboratory procedures for fresh flayed goats skins

The cutting from each piece of freshly flayed goat skin were then soaked in some volume (that is 20ml; volume/weight) of sterile physiological buffered saline (PBS) and allowed to stay for thirty minutes with shaking at intervals to ensure maximum extraction of the microfloral from the pieces of skin into the extracting fluid.

After thirty minutes, each piece was lifted and allowed to drain freely into the bottle. The extracting fluid from the five sample bottles were pooled into another two-hundred millilitre sterile bottle. It was then mixed thoroughly but gently.

About twenty millilitre (20mls) was decanted into sterile centrifuge tube and centrifuged at 2,500 revolutions per minutes (2,500 r.p.m.) for ten minutes. The supernatant was decanted and the sediment was shaken with the last few drops of the supernatant to redissolve. This was then used for the isolation and identification procedures.

Using sterile test tubes, the sediment was divided into two equal halves. To the first half, an equal volume of two percent phenol was added to get rid of the vegetative form of other bacteria other than spore forming bacteria. This was allowed to stand at room temperature for thirty minutes with slight shaking at intervals. (Spores of Bacillus anthracis are resistant to 2% phenol while other non-spore forming micro-organisms are sensitive (George, 1978).

The second half was put in a water bath with a temperature of 60°C for thirty minutes with slight shaking at intervals to ensure uniform temperature

of 60°C with slight shaking at intervals for thirty minutes.

Laboratory procedures for scientifically-tanned leather samples

Each piece of the scientifically tanned leather collected was trimmed to an area of 20sq. cm and soaked in 20ml of PBS, for thirty minutes with slight shakings at intervals. The extracting fluid for each set was pooled into a sterile centrifuge tube and centrifuged for ten minutes at 2,5000 r.p.m.

The supernatant was decanted off leaving few drops to dissolve the sediments on shaking and divided into two equal halves. An equal volume of 2% phenol was added to the first half, allowed to stay for another thirty minutes with slight shakings at interval. While the second half was also put in a water bath at 60°C for thirty minutes with shaking at intervals.

Bacteriological examinations of field isolates

(i) Isolates from raw goats skins

The treated sediments were then inoculated separately on the following bacteriological media

for isolation purposes.

(a) Blood agar (BA): - containing 7% defibrinated sheep blood.

(b) Phenol-ethanol agar (PEA) - containing 7% defibrinated sheep blood.

The inoculated agar plates were incubated at 37°C in candle jars to create a condition of about 10--20 percent carbon dioxide and 80--90 percent oxygen for about twenty-four hours.

The BA and PEA plates were examined for typical colonies of Bacillus anthracis. The colonies selected for further investigations were the fairly-large, dry, greyish, non-hemolytic, rough colonies with serrated edges ('medusa-head'), typical for B. anthracis. Suspected B. anthracis colonies were re-inoculated onto fresh BA and PEA plates and incubated at 37°C in total aerobic conditions for isolation into pure cultures and maximum growth.

Staining

The isolates were stained by Gram and Anthony (Modification of Hiss) stains for their gram and capsular reactions respectively. The stained slides were examined under a simple microscope to observe the staining reactions and cell-morphology.

Biochemical characterization

The biochemical identification procedures were carried out according to standard techniques (Table 3:1 shows typical reactions of B. anthracis).

The isolates behaving typically like B. anthracis were then inoculated onto stock-culture agar slants and incubated at 37°C for 24 hours.

These were subsequently kept as stock cultures in the refrigerator (4°C) until further tested.

(ii) Isolates from locally-tanned leather samples

The treated sediments in the two tubes were then inoculated each on BA and PEA plates for cultural examinations. The plates were incubated at 37°C in candle jars (creating an atmosphere of about 10-20% CO_2 and 80-90% CO_2) for twenty-four hours.

After incubation, the BA and PEA plates were examined for typical B. anthracis colonies. The suspected isolates were then re-inoculated onto BA and PEA plates for pure culturing at 37°C for 24 hours. Isolates were tested biochemically as

already stated above and th se behaving typically like B. anthracis were stored at 4°C for further testing.

(iii) Isolates from scientifically-tanned leather samples

The two halves, after been treated as mentioned above, were each inoculated on both BA and PEA plates for cultural characterization. The plates were incubated at 37°C in candle jars (10-20% CO_2 + 80-90% O_2) for 24 hours.

After incubation, the plates were observed for typical growth of B. anthracis. The suspected isolates were re-inoculated on fresh BA and PEA plates to give pure cultures for biochemical characterization and then stored for further investigations.

(B) Animal Pathogenicity Test

Total number of isolates tested

Twenty-six isolates were found to be typically, behaving like Bacillus anthracis. Eleven of them were from the freshly flayed goats skins, eight

Table 3:1 Typical Biochemical Reaction of *Bacillus anthracis*

Biochemical Tests	*Bacillus anthracis* Reactions
Gram staining	Gram positive
Motility	Non-motile
Capsular staining	Capsulated
Spores	Oval, do not swell cell
Simmon's citrate	Positive
Nitrate Reduction	Positive
Gelatin Hydrolysis	Weak
Urea-christensens	Variable (+ or -)
Voges - Proskauer	Positive
Glucose	Acid production
Gas from glucose	Negative
Mannitol hydrolysis	Negative
Arabinose	Negative
Action on blood	Non-hemolytic
Growth at 25°C	Positive
Growth at 37°C	Positive
Growth at 42°C	Variable (Positive/Negative)

Source: 1. Cowan, and Steel, 1970,
2. . King, 1972,
3. . Carter, 1973.

from the locally-tanned goats leather and the rest seven from the modern or scientifically-tanned leather samples.

Source of mice

One-hundred and fifty growing swiss white mice were purchased for the animal inoculation tests. Ninety of them were males and sixty females. Five mice containing at least two males and two females were used for each isolate.

The mice were obtained from two sources. The bulk of them was from the National Veterinary Research Institute (NVRI) in Vom-Jos, Plateau State of Nigeria and the rest from the Department of Veterinary Pathology and Microbiology, Ahmadu Bello University, Zaria.

They were kept in large mice cages for one week, for their general observation in an isolated, disinfected room. They were fed on mice pellets and drank freely from water bottles.

Preparation of cultures

Each isolate from the stock-culture was inoculated on brain heart infusion (BHI) broth

and incubated at 37°C for 24 h. This was to reactivate the organisms before inoculating them into the mice.

Inoculation of mice

Using a sterile needle and a syringe, about 0.2ml of twenty-four hours fresh culture of the isolate was injected intraperitoneally into the five mice. The five inoculated mice for each isolate were kept together in a cage with feeds and clean water, and were observed for 72 h post inoculation. Usually, injection of virulent B. anthracis will cause death within sixteen to forty-eight hours. If injected mice were not dead at the end of seventy-two hours, the suspected organism was probably not a virulent B. anthracis (George, 1978).

Inoculated animals that died within the stipulated time were picked and put in sterile wide mouth and well-corked sample bottles for post mortem examinations.

Examination of dead mice

The inoculated mice that died within 18-48 hours were examined at necropsy for pathologic

lesions. The antopsies were done in a dissecting tray liberally disinfected with 1:1000 mercuric chloride. The dissecting kits were also sterilized by autoclaving and sometimes by cold sterilization using acetone and alcohol. In addition, swabs of the peritoneal exudate for each dead mouse were made for bacteriological examination.

Bacteriological examination

Smears of the peritoneal exudate were made on clean glass sides from a swab. The and capsular staining of the smears were done for their respective and capsular reactions.

The other swab was then used to streak on BA and PEA plates and incubated aerobically at 37°C for twenty-four hours.

After incubation, colonies looking typical of B. anthracis colonies were stained by the Gram and Anthony staining techniques.

Control Experiments

Use of reference strains of Bacillus anthracis

Pathogenic and vaccine strains of B. anthracis were obtained from National Veterinary Research

Institute (NVRI), Vom-Jos, Plateau State, Nigeria.

The vaccine strain was in the lyophilized form. It was reconstituted with sterile PBS and then inoculated into nutrient broth, incubated at 37°C for twenty-four hours, after which they were observed for growth.

The cultures of vaccine and the pathogenic strains were then inoculated on to BA and PEA plates. These were incubated at 37°C for 24h, and typical colonies of B. anthracis were obtained in both cases.

These reference strains were used along-side with isolates in the survey and in the mice pathogenicity tests. The results obtained here were compared with the ones obtained for the isolates from the survey.

Use of other species of Bacillus

Five isolates of Bacillus species were used in this study. Four isolates obtained during the survey behaved typically like B. cereus and one other isolate behaved like B. subtilis. They were used in mice pathogenicity tests and the results obtained were compared with those of the other isolates that behaved typically like B. anthracis.

Survival of tanning process by Bacillus anthracis

This experiment was done to determine the survival of Bacillus anthracis through tanning processes. For the experiment, six randomly picked isolates, two each from the raw goats skins, locally and scientifically-tanned leathers were first used. In addition, the reference strain of pathogenic B. anthracis from NVRI, Vom-Jos was used.

Two pieces of freshly flayed goat skins were collected from the slaughter house in Samaru-Zaria. They were examined for the presence of B. anthracis as already described above. From the bacteriological examinations, they were certified to be free of B. anthracis organism. The skins were then used for the tanning processing.

Two pieces of flayed goat skin of equal size (10 x 10cm) were cut and weighed. They were washed thoroughly with soap and rinsed in clean water before further use. One of them was used as the experimental and the other, the control. One piece was seeded with two loopful pure cultures of the isolate and the other piece with reference pathogenic strain of B. anthracis.

These seeded pieces were incubated aerobically at 37°C in sterile petridishes for twenty-four hours. After incubation, the seeded experimental piece was soaked in tap water. The control piece was soaked in 1:1000 mercuric chloride for five minutes (to kill off the Bacillus anthracis (Solyts, 1948) then washed thoroughly with tap water. Fluids and swabs from the two pieces were then taken and streaked on blood agar plates, incubated at 37°C for twenty-four hours.

The pieces of skin were then tanned concurrently in separate covered jars.

The chrome-tannage was employed for the tanning and the steps involved were in accordance with the standard technique (Appendix II).

a. Weighing:- The pieces of skin were weighed to know the amount of the chemical needed for liming.

b. Washing:- The skins were washed with distilled water

c. Liming or pasting:- The chemical used were a mixture of

distilled water per weight of skin. The skins were left in this solution for one and a half hour.

h. Washing:- The skins were again washed in distilled water

i. Bating:- The chemicals used here were:- 1% bate powder per weight of skin, plus 150% distilled water per weight of skin. The mixture had a final pH of 8 and the skins were left in here for only thirty minutes.

j. Pickling:- The chemicals used were 70% distilled water per weight of skin, plus 10% sodium chloride per weight of skin plus 1% concentrated sulphuric acid per weight of skin.

The mixture gave a final pH of 1.5 and the pieces of skin were soaked in here for thirty minutes.

k. Tanning:- Seven percent chrome powder dissolved in distilled water

calculated per weight of the skin and a pH of 2 was used, with the skins soaked in there for three hours.

1. Retanning:- Another seven percent chrome powder per weight of skin dissolved in distilled water was used. The skins were soaked in here overnight.

This was the line up of the tanning procedure followed. The other subsequent steps including horse-up, neutralisation, fat-liquoring and finishing had to be omitted because the machineries were not available in the laboratory.

At the end of each step in the tanning process, some quantity of the fluid and a swab of the skin was taken. The fluids and the swabs were used to streak on the surfaces of BA and PEA plates, and incubated at $37^{o}C$ for 24 hours.

The incubated plates were then observed for possible growth of surviving seeded B. anthracis.

The suspected isolates were then tested by staining and biochemical reactions.

Chapter Four

RESULTS

(A) Prevalence of Bacillus anthracis in freshly flayed goat skins

Out of the 102 samples of freshly flayed goat skins surveyed for B. anthracis, a total of 51 isolates (50%) yielded Bacillus species while the rest 51 of them (50%) were negative.

Other bacteria, mostly gram-positive cocci, in short chains, and or clusters and few gram-variable cocco-bacilli were observed from them.

The results of biochemical tests done to differentiate the Bacillus species showed that 11 of them were suggestive of being B. anthracis while the remaining forty were of non-anthrax bacilli, (Appendix V).

Table . shows the biochemical reactions of the reference strains of B. anthracis and the 11 isolates from fresh flayed skins.

Table 4:1 Biochemical reactions of reference strains of Bacillus anthracis and the suspected isolates from fresh flayed goat skins

Samples number	Gram Reation	Capsular Reaction	Motility	Citrate	Urea	Nitrate	Voges Proskauer	Glucose	Arabinose	Mannitol	Gelatin liquefaction
Virulent strain	Gm +ve long rods	CS	-	+	-	+	+	A	-	-	S
Avirulent strain	"	CS	-	+	-	+	+	A	-	-	S
RS 2	"	CS	-	+	-	+	+	A	-	-	S
RS 3	"	CS	-	+	-	+	+	A	-	-	S
RS 51	"	CS	-	+	-	+	+	A	-	-	S
RS 58	"	CS	-	+	-	+	+	A	-	-	S
RS 66	"	CS	-	+	-	+	+	A	-	-	S
RS 71	"	CS	-	+	-	+	+	A	-	-	S
RS 72	"	CS	-	+	-	+	+	A	-	-	S
RS 82	"	CS	-	+	-	++	+	A	-	-	S
RS 89	"	CS	-	+	-	+	+	A	-	-	S
RS 90	"	CS	-	+	-	+	+	A	-	-	S
RS 102	"	CS	-	+	-	+	+	A	-	-	S

FOOTNOTE:
RS = Raw skin sample
CS = Capsulated with spores
Gm tve = Gram positive
\+ = Positive reaction
\- = negative reaction
A = Acid production
S = Slow gelatin liquefaction

2. Prevalence of B. anthracis in locally tanned Leather

Out of the 103 samples collected for this survey, 12(12%) yielded Bacillus species.

The other isolates were gram positive cocci, cocco-bacilli and few gram variable cocci and coccobacilli. Eight of the 12 suspected Bacillus species gave typical biochemical reactions of B. anthracis (Table 4:2). The other four were non-typical (Appendix VI).

3. Prevalence of B. anthracis in scientifically tanned leather

Out of the 103 samples collected for this survey, 10(9.7%) of them contained Bacillus species. Seven of them showed reactions like the typical anthrax bacillus (Table 4:3). The remaining samples contained both gram positive and gram negative cocci and cocco-bacilli (Appendix VI).

(B) Results of animal pathogenicity tests

(i) Field isolates of B. anthracis

Out of the first 11 isolates from fresh flayed goat skins, 9 (9%) of them killed the mice within

Table 4:2 Biochemical reactions of suspected Bacillus anthracis isolated from locally-tanned goat leather

Samples Number	Gram reaction	Capsular Reaction	Motility	Citrate	Urea	Nitrate	Voges Proskaeuer	Glucose	Abrabinose	Mannitol	Gelatin liquefaction
LL 7	Gm tve long rods	CS	-	+	-	+	+	A	-	-	S
LL 17	"	CS	-	+	-	+	+	A	-	-	S
LL 28	"	CS	-	+	-	+	+	A	-	-	S
LL 29	"	CS	-	+	-	+	+	A	-	-	S
LL 48	"	CS	-	+	-	+	+	A	-	-	S
LL 90	"	CS	-	+	-	+	+	A	-	-	S
LL 101	"	CS	-	+	-	+	+	A	-	-	S
LL 103	"	CS	-	+	-	+	+	A	-	-	S

FOOTNOTE: LL = Locally-tanned leather
Gm tve = Gram positive
CS = Capsulated with spores
S = Slow gelatin liquefaction
+ = Positive reaction
- = Negative reaction
A = Acid Production

Table 4:3 Biochemical reactions of suspected Bacillus anthracis isolated from scientifically-tanned leather

Samples Number	Gram Reaction	Capsular Reaction	Motility	Citrate	Urea	Nitrate Reduction	Voges Proskauer	Glucose	Arabinose	Mannitol	Gelatin liquefaction
SFL 4	Gm +ve Long rods	CS	-	+	-	+	+	A	-	-	S
SFL 23	"	CS	-	+	-	+	+	A	-	-	S
SFL 56	"	CS	-	+	-	+	+	A	-	-	S
SFL 59	"	CS	-	+	-	+	+	A	-	-	S
SFL 87	"	CS	-	+	-	+	+	A	-	-	S
SFL 101	"	CS	-	+	-	+	+	A	-	-	S
SFL 102	"	CS	-	+	-	+	+	A	-	-	S

FOOTNOTE: SFL = Scientifically finished (tanned) leather
Gm +tve = Gram positive
CS = Capsulated with spores
\+ = Positive reaction
\- = Negative reaction
A = Acid production
S = Slow gelatin liquefaction

Table 4:4 Biochemical reaction and animal pathogenicity tests of other Isolated species of Bacillus other than Bacillus anthracis

Bacillus species	Gram Reaction	Capsular Reaction	Motility	Citrate	Urea	Nitrate	Voges Proskaeur	Glucose	Arabinose	Mannitol	Gelatin Liquefaction	Animal Pathogenicity tests
B1	Gm +ve long rods	CS	+	+	+	+	+	A	-	-	R	No death occured
B2	"	CS	+	+	+	+	+	A	-	A	R	" "
B3	"	CS	+	+	+	-	+	A	-	-	R	" "
B4	"	CS	+	+	+	+	+	A	-	-	R	" "
B5	"	CS	+	+	+	+	+	A	-	A	R	" "

FOOT NOTE: B1 - B5 = Bacillus species 1-5 (suspected)
Gm +ve = Gram postive long rods
CS = Capsulated with spores
+ = Positive reaction
- = Negative reaction
A = Acid production
R = Rapid in gelatin liquefaction (all at 72 hr.)

(B) RESULTS OF ANIMAL PATHOGENICITY TESTS

i. Field isolates of B. anthracis

Out of the first 11 isolates from fresh flayed goat skins, 9 (9%) of them killed the mice within the recommended 18 and 48 hours. From the locally-tanned all 8 (100%) isolates killed the mice at the end of the 48 hours post inoculation while for the scientifically tanned, 6 (85.7%) isolates out of 7 killed the mice inoculated with them within the 18th and 48th hour post inoculation. (Appendix v).

ii. Reference strains of B. anthracis

The reference strains of pathogenic and vaccine strains obtained from NVRI, Vom-Jos showed the same biochemical reactions typical of B. anthracis.

For the animal pathogenicity tests, all the five mice inoculated with the pathogenic strain died within the 18-48 hour post inoculation. None of the mice inoculated with the vaccine strain died within 18-48 hours post inoculation (Appendix iv).

iii. Other Bacillus species

The results of biochemical reactions of five non-anthrax bacilli and their animal pathogenicity tests are shown below in Table 4:4. None of the five isolates killed any of the mice in seven days post inoculation. The mice were killed after and examined bacteriologically and pathologically. No bacillus was recovered from the peritoneum and there were no gross lesions.

GROSS PATHOLOGICAL AND BACTERIOLOGICAL FINDINGS IN DEAD MICE

Gross pathological lesions

Grossly, the mice were generally bloated. Some of them were in advanced stage of decomposition especially those which died in the evening and were not noticed until the following day. The peritoneum of each mouse contained blood tinged exudate. The organs of the peritoneal and thoracic cavities were oedematous and or haemorrhagic.

Bacteriological findings

Gram positive bacilli were demonstrated by direct microscopic examinations of Gram stained

exudates from the mice. Capsules were also demonstrated from some of them.

Culturally, the bacterium was also isolated from the exudates when cultured on BA and PEA plates for 24h at 37°C under aerobic conditions.

Survival of B. anthracis during tanning process

It was observed that B. anthracis survived exposure to 1:1000 mercuric chloride for five minutes. However, when the period of soaking was extended to thirty minutes, bacteriologic examinations showed that there was no viable organism on the piece of skin.

In general, all the tanned pieces of skin seeded with the isolates from survey and the pathogenic strain of B. anthracis contained viable anthrax organisms from the first step of washing to the bating stage. But, at the pickling stage, no anthrax organism or other bacteria were isolated.

Chapter Five

DISCUSSION

The survey of B. anthracis in locally-tanned leather showed that 7.3 percent of the samples collected contained virulent strains of the organism. This is quite high enough to cause detrimental effects for both the tanners and consumers or users of their tanned leathers and products.

Equally of public health significance was the finding that scientifically-tanned leathers may also be contaminated by B. anthracis. This is because there was no significant difference in the carriage rate of B. anthracis in scientifically and locally-tanned leathers. It has been found that some highly resistant spore-forming organisms especially B. anthracis can survive through the beam houses of leather, hairs and wool (Vekatesan, 1979).

Data obtained from experimentally inoculated raw skin subsequently exposed to scientific method of tanning in the present study, however demonstrated that the process used did not allow survival of

B. anthracis (1 cal strains) at or beyond the pickling stage. All bacteria and twenty-two other microorganisms were eliminated at the pickling stage. The finding that scientifically-tanned leather were contaminated by B. anthracis in this environment is therefore indicative of post-process contamination. The air-borne spores of B. anthracis may readily contaminate finished and stored products. To date, it is unknown whether the local tanning process allows survival of B. anthracis.

The population of goats in Nigeria is approximately between 27-32 million (Bida, 1979). Since most herdsmen rear their animals together, it could be speculated that the cattle and sheep herds are equally involved in carrying the spores of B. anthracis and exchanging skin microorganism by close contact and herding.

Although the percentages of samples containing viable virulent B. anthracis in both locally and scientifically tanned leathers in the present survey are relatively low, these could be a reflection of narrow scope of the study. Isolation of virulent B. anthracis from tanned leathers to which the general human population is exposed is

without doubt significant.

Before any recommendation on immunization of tannery workers and herdsmen a broad-based study covering many states in Nigeria will have to be embarked upon to definitely assess the degree of the problem posed to human health in Nigeria by raw skin and tanned leather.

Of laboratory safety significance was the finding that some strains of virulent B. anthracis survived a treatment of five minutes in 1:1000 mercuric chloride which is the standard procedure for disinfection. The present study has demonstrated that a period of thirty minutes is needed to ensure adequate contact and total destruction of infectious organisms. The exposure potential of investigators working with B. anthracis using the recommended five minutes in 1:1000 mercuric chloride cannot be over-emphasized. Also, tannery effluents from both local and modern tanneries should be disposed off safely to prevent possible health hazards from effluents contaminated with spores of B. anthracis and or surviving vegetative cells.

CONCLUSION

Bacillus anthracis is a deadly organism of warm blooded animals including man. It has been found to be one of the highly resistant spore-forming organisms posing threats to our beam-houses and tanneries.

Economically, good, safe leather products can boost the country's economy as regards export earning. So, enough and adequate care must be taken to ensure a good control of anthrax in the country.

People dealing with raw and dry unprocessed hides and skins should avoid dusting during transportation, packing, unpacking, sorting and handling of these materials, animal hairs bristles and wool. Tannery workers should protect the slightest skin injury, wear protective clothings, wash their bodies carefully and disinfect working cloths after leaving the tanneries.

At this stage it is not known if B. anthracis are recoverable from tannery pits, where the effluent flowsinto until disposed. It is recommended that a bacteriological check should be conducted in order to assess the possible

environmental pollution hazard posed by the present methods of disposal of effluents from leather industries.

More work need to be done to determine the prevalence of this deadly disease, anthrax, in our society, especially in the herds and beam-houses including tanneries. This is most important in relation to Nigerian economy as regards importation of leather products and exportation of raw, dried and half-way tanned hides, skins and their leather products.

REFERENCES

Albrink, W. S. (1961). 'Pathogenesis of inhalation anthrax' Bacteriological Review. 269-273.

Al-dulaimy, S. B. and Al-allaf, G. A. (1978). "Anthrax meningitis: Case Report; Transactions for Royal Society of Tropical Medical Hygiene. 72: 315.

Aleksandrov, N. I., Gefen, N. E., Gapochko, K. G., Gain, N. S., Sargeyev, N. M., Lazareva, E. S., Mishchenko, V. V. and Shlyakhov, E. N. (1961). Aerosol immunization with live dried vaccines and toxoids VI. A study of post-vaccination reaction and immunological efficacy of aeroso-immunization with aerosol brucellosis, tularemia, anthrax and plaque vaccines in man. Journal of Microbiology and Epidemiology. 32: 1245-1252.

Anderson, (1945) - Cited by Venkatesan, R. A. (1979).

Angelety, L. F. and Wright, G. G. (1971). 'An agar diffusion method for the differentiation of Bacillus anthracis' Applied Microbiology. 21: 157-159.

Anon (1963) - Cited by Venkatesan, R. A. (1979).

Anonymous (1967). "Anthrax: A problem of the livestock industry". Veterinary Record 85, 89.

Arion (1965) - Cited by Merchant and Parker (1971).

Auerbach, S. and Wright, G. G. (1955). 'Studies of immunization to anthrax VI. Immunizing activity of protective antigen against various strains of Bacillus anthracis, Journal of Immunology, 75: 129-135.

Beaton, W. G. (1939). 'Reported cases of anthrax' Nigerian Annual Report of Veterinary Department, p. 31.

Belton, F. C. and Henderson, D. W. (1956) "A method for assaying anthrax immunizing antigen and antibody", British Medical Journal, 37: 156-160.

Bida, S. A. (1975). "Paper presented at a Symposium". Leather as a foreign exchange earner for a developing nation, Leather Research Institute, Zaria.

Biegeleson, J. Z., Jr., Chemy, W. B., Skaliyipag and Moody, M. D. (1962). "The demonstration of Bacillus anthracis in environmental specimens by conventional and fluorescent antibody techniques". American Journal, 75: 230-239.

Blank, J. (1937). "The action of micro-organisms on animal skins". Journal of American Chemists Association, 32: 294.

Bormans (1952) - Cited by Merchant and Parker (1971).

Brachman, P. S., Gold, H., Plotkin, S. A., Fekety, F. M., Wein, M. and Ingraham, N. R. (1962). "Field evaluation of a human anthrax vaccine". American Journal for Public Health; 52: 632-645.

Buchanam, T. M., Feeley, J. C., Hayes, P. S. and Brachnan, P. S. (1975). "Anthrax indirect microhemagglutination test". Journal of Immunology, 107: 1631-1636.

Burdon, K. L. (1956). "Useful criteria for the identification of Bacillus anthracis and related species". Journal of Bacteriology, 71: 25.

Burdon, K. L., and Wende, R. D. (1960). "On the differentiation of anthrax bacilli from Bacillus cereus". Journal of Infectious Diseases, 107: 224.

Burrows, W. (1973). "Textbook of Microbiology". (20th Edition), W. B. Sanders Company, Philadelphia, Pg. 607-614.

Calvin, W. A. (1969). 'Veterinary Medicine and Human Health', (2nd edition), Baltimore, U.S.A. The Williams and Wilkins Company, Pg. 89-615.

Campbell, A. D. (1955). 'Reported cases of anthrax', Eastern Region of Nigerian Annual Report of Veterinary Department, pg. 3.

Campbell, A. D. (1956). 'Reported cases of anthrax', Eastern Region of Nigerian Annual Report of Veterinary Department, p. 12.

Carter, G. R. (1973). Diagnostic Procedures in Veterinary Microbiology. 2nd Ed. C. C. Thomas Springfield, Ill, U.S.A. p. 120-124.

Chamberland and Roux (1959) - Cited by Merchant and Parker (1971).

Cordon, T. C., Everett, A. L. and Windus, W. (1961), 'Elastate activity of some enzymes as related to their depiletory action'. Journal of American Leather Chemists Association, 56: 68.

Cowan, S. T. and Steel, J. K. (1970), 'Manual for the identification of Medical Bacteria, Cambridge University Press.

Collet, 1923-1926 - Cited by Venkatesan, R. A. (1979), Journal of Society of Leather Technologists and Chemists, 63: 55.

Cromatie, W. I. (1947), 'Anthrax', Journal of Infectious Diseases, 80: 1-153.

Davaine, 1863-1864 - Cited by Hubbert et al.

Davies, D. G. and Harvey, R. W. S. (1972), 'Anthrax infection in bone meal from various countries of origin. Journal of Hygiene, Cambridge, 70: 455.

De-Armon, I. A. Jnr. (1961). 'Immunological studies of anthrax, I. An index to determine quantitative immunization, Journal of Immunology, 87: 233-239.

Gaiger (1959) - Cited by Merchant and Parker (1971)

George, E. O. (1978). 'Manual of Standardised methods for Veterinary Microbiology, Cornell, University Press, Washington.

Gibsons, T. (1963). 'An investigation of the Bacillus group, Journal of Bacteriology, 28: 295-313.

Gladstone, (1946) - Cited by Merchant and Parker (1971).

Gold, H. (1967). 'Anhtrax, A report of one hundred and seventeen cases; Journal of Bacteriology, 96: 387-396.

Hanson, R. P. (1959). 'The earliest account of anthrax in man and animals in North America', Journal of American Veterinary Medical Association, 135: 463-465.

Hubbert, W. T., McCulloch, W. F. and Schnurrenberger, P. R. C. (1975). 'Diseases transmitted from animals to man', Charles C. Thomas (Publisher), Illnois, U.S.A. Pg. 237-250.

Heyworth, B., Voos, U. G., Ropp, M. E. Meinel, H. I. and Darlow, H. M. (1975). 'Anthrax in the Gambia, an epidemiological study; British Medical Journal, 4, 5988: 79-82.

Jarolimek, (1960) - Cited by Venkatesan, R. A. (1979).

Jovanovitis (1920) - Cited by Venkatesan, R. A. (1979).

King, E. O. (1972) - 'The identification of unusual pathogenic Gram-negative bacteria.' Preliminary Revision by Robert, E. Weaver et al. July (1980). Pg. 12. CDC Atlanta, Georgia, U.S.A.

Klein, F. (1963). 'Dual nature of resistance mechanisms as revealed by studies of anthrax septicemia', Journal of Bacteriology, 85: 1032-1038.

Klein, F., De-armon, I. A. Jnr., Lincoln, R. E., Mahilanatt, B. C. and Fernelius, A. L. (1962). 'Immunity against Bacillus anthracis from protective antigen live vaccine', Journal of Immunology, 88: 15.

Klemm, D. M. and Klein, W. R. (1959). 'History of anthrax: Journal of American Veterinary Medical Association 135: 458-462.

Knight, A. H., Wyne-Williams, C. and Willis, A. T. (1969). 'Cutaneous anthrax in the non-industrial hazard', British Medical Journal, 1: 416-18.

Koch (1874) - Cited by Hubbert et al. (1975).

Koppenchoefer, R. M., and Somer, G. L. (1939). 'The curing of heavy hides', Journal of American Leather Chemists Association, 34: 34.

Larforce, F. M., Bumford, F. H., Feeley, J. C., Stokes, S. L. and Snow, D. B. (1970). 'Epidemiologic study of a fatal case of inhalation anthrax, Archives Environmental Health, 18: 798-805.

Lamanna, C., Eisler, D. (1960). 'Epidemiology of anthrax bacilli, Journal of Bacteriology, 79: 4358.

Lamb, R. (1973). 'A new look at infectious diseases; Anthrax, British Medical Journal, 1, 5846: 157-160.

Leise, J. M., Carter, C. H., Friedlander, H. and Freed, S. W. (1959). 'Anthrax', Journal of Bacteriology, 77: 665.

Leise, J. M., Carter, C. H., Friedlander, H. and Freed, S. W. (1959). 'Criteria for the identification of Bacillus anthracis', Journal of Bacteriology, 77: 655-660.

Line (1948) - Cited by Venkatesan, R. A. (1979).

Llyod, D. H. (1971). 'West African bovine streptothricosis', Span 14: 1-4.

Lyon, D. G. (1973). 'An outbreak of anthrax of the Chester Zoological Gardens. Veterinary Records, 92, 13: 334-337.

Martin (1957) - Cited by Venkatesan, R. A. (1979)

Martin, C. M., Mognusson, S., Goscienski, P. and Hansen, C. F. (1961). 'Common human viruses as carcinogen vectors. Science, 134: 1985-1986.

MacFadyen, J. A. (1903). 'A peculiar staining reaction of the blood of animals dead of anthrax. Journal of Comparative Pathology and Therapy. 16: 35-40.

McGann, U. G., Stearman, R. L. and Wright, G. G. (1961). 'Studies on Immunity to anthrax VIII. Relationship of complement fixing activity to protective activity of culture filtrates. Journal of Immunology, 86: 458-464.

Mclaughlin, Rockwell and Blank (1927) - Cited by Venkatesan, R. A. (1979).

Merchant, I. A. and Parker, R. A. (1971). 'Veterinary Bacteriology and Virology', (7th edition), The Iowa State University Press, Ames, Iowa, U.S.A. Pg. 386-397.

Minnet, F. C. C. (1952). 'Sporulation and viability of Bacillus anthracis in relation to environment, temperature and humidity, Indian Veterinary Journal, 28: 326-340.

Morris, E. J. (1955). 'A selective medium for Bacillus anthracis', Journal of General Microbiology, 13: 456-460.

Nalin (1967) - Cited by Burrows (1973).

Nandy, I. K., Sen, I. and Das J. (1976). 'Symposium on East India. Tanning Industry and Tanning agents, Central Leather Research Institute, Madras, Pg. 269.

Nopitsch (1954) - Cited by Venkatesan, R. A. (1979) "Microbiology in Leather Manufacture". Journal of the Society of Leather Technologists and Chemists, 63: 55.

O'Flaherty, R., Roddy, A. and Lollar, H. (1965). "Chemistry and Technology of Leather". Vol. 1, New York, Rainhold, p 121-125.

Pasteur (1935) - Cited by Merchant and Parker (1971).

Pericaud (1925) - Cited by Venkatesan, R. A. (1979).

Pienaar, U. and De, V. (1967). "Epidemiology of anthrax in wild animals and the control of anthrax epizootics in the Kouger National Park, South Africa", Fed. Proc. 26: 1496-1502.

Prevot, J. (1959-1960). "The microflora of tanning liquors, and of vegetable tanned eather", Journal of American Leather Chemists Association. 55: 542.

Prey, J. (1925). "Effect of addition of sodium sulphide times upon solution of nitrogen from hides and hair". Journal of American Leather Chemists Association. 20: 273.

Procter, M. (1922). "The Principles of Leather Manufacture". (2nd edition), E. and F. N. Spon. Limited, London. p. 236.

Puziss, M. and Wright, G. G. (1963). "Studies on immunity of anthrax IX. Gel-adsorbed protective antigen for immunization of man". Journal of Bacteriology. 84: 230-236.

Ray, J. G. Jnr., and Kachell, P. J. (1964). "Agar-gel precipitin technique in anthrax antibody determination". Applied Microbiology, 12: 249-354.

Rayer, (1850) - Cited by Hubbert et al (1975) "Diseases transmitted from animals to man". p. 242.

Roddy (1964) - Cited by Venkatesan, R. A. (1979).

Schlingman, A. S., Devlin, H. B., Wright, G. G., Maire, R. J., and Manning, M. C. (1956) - 'Immunizing activity of alum precipitated protective antigen of Bacillus anthracis in cattle, sheep and swine', American Journal of Veterinary Research, 17, 256-261.

Sen and Minett (1956) - Cited by Burrows (1973).

Smith, I. M. (1973). 'A brief review of anthrax in domestic animals', Post-Graduate Medical Journal. 49, 574: 571-572.

Solyts, (1963) - Cited by Merchant and Parker (1971).

Stanley, J. L. and Smith H. (1961). 'Purification of Factor 1 and recognition of a 3rd factor of the anthrax toxin', Journal of General Microbiology, 26: 49-66.

Stein, C. D. (1963). Anthrax, 'Diseases transmitted from animals to man', (5th edition), T. G. Hull, Springfield, C. C. Thomas Pg. 82-125.

Stein and Rogers (1946) - Cited by Merchant and Parker (1971).

Stein, C. D. and Van-ness, G. B. (1955). 'A ten year survey of anthrax in livestock with special reference to outbreaks in 1954. Veterinary Medicine. 50: 519-588.

Sterne, M. (1959). 'Diseases due to bacteria. In "Infectious Diseases of Animals". Stable-forth A. W. Galloway, I. A. London, Butterworth Scientific Publications, Vol. I.

Sterne, M. (1967). 'Distribution and economic importance of anthrax - cited by Hubbert et al. (1975) - Diseases transmitted from animals to man. Pg. 242.

Stiles, G. W. (1944). 'Isolation of Bacillus anthracis from spirose ear-ticks: (Ornithodorus megnini). American Journal of Veterinary Research 5: 318-319.

Strange, R. E. and Beiton, F. C. (1954). "Studies on a protective antigen produced in vivo from Bacillus anthracis. Purification and chemistry of the antigen". British Journal for Experimental Pathology 35: 153-165.

Strange, R. E. and Thorne, C. B. (1958). "Further purification studies of the protective antigen of Bacillus anthracis produced in vivo". Journal of Bacteriology. 76: 192-202.

Stuart, L. S. (1940). "Studies on the resalting of salt-cured calfskins". Journal of American Leather Chemists Association. 35: 554.

Sulkin, S. E., Long, E. R., Pike, R. M., Sigel M. M., Smith, L. E. and Wedum, A. G. (1963). "Laboratory infection and accidents". In Harris, A. H. and Coleman, M. B. (Eds.) "Diagnostic Procedures and Reagents". 4th Edn. New-York, American Public Health Association Incorporation. p 89-104.

Taylor, L. and Carslaw, R. W. (1967). "Cutaneous anthrax". Lancet, 1214-1216.

Taylor, M. J., Kennedy, G. H. and Bludell, G. P. (1961). "Experimental anthrax in rat I. The rapid increase of natural resistance observed in young hosts". American Journal of Pathology. 38: 469-480.

The Holy Bible, Exodus Chapter (7:1-7).

Thorne, C. B., Gomez, C. G. and Houseweight, R. D. (1960). "Synthesis of glutamic acid and glutamyl polypeptide by Bacillus anthracis II. The effect of carbon dioxide on peptide production in solid media". Journal of Bacteriology, 63: 363-368.

Thorstensen, T. (1969). 'Practical Leather Technology', Van, Nostrand, Reinhold Pg. 35-40.

Vankatesan, R. A. (1979). 'Microbiology in Leather Manufacture', Journal of Society for Leather Technologists and Chemists, 63: 55-66.

Ward, M. K. (1965). 'Studies on anthrax infection in immunized guinea pigs. Journal of Infectious Diseases, 115: 59-67.

Wilson, G. S. and Miles, A. A. (1964). 'Principles of Bacteriology and Immunity (5th edition) Baltimore. Williams and Wilkins P . 2079-2094.

Wilson, J. B. and Rusell, K. E. (1964). 'Isolation of Bacillus anthracis from soil stored for sixty years, Journal of Bacteriology, 87: 237-238.

Wolff, A. and Heimann, H. (1951). 'Industrial anthrax in the United States of America. American Journal of Hygiene. 53: 80-109.

Wolfgang, K. J. (1968). 'Zinsser Microbiology' (16th Edition).

World Health Organization (1967). 'Technical Report Series 378. Joint FAO/WHO Expert Committee on Zoonoses, 3rd Report, Pg. 48-54.

Wright, G. G. (1954). 'Studies on immunity in anthrax III. Elaboration of protective antigen in a chemically defined non-protein medium'. Journal of Immunology, 72: 263-259.

Wright, G. G., Green, T. W., and Kanode, R. G. Jnr. (1954). 'Studies on immunity in anthrax V. Immunizing activity of alum-precipitated. Journal of Immunology, 73: 387-391.

Young, L. S., Feeley, J. C, and Brachman, P. S. (1970). 'Vaporized formaldehyde treatment of a textile mill contaminated with Bacillus anthracis, Architect for Environmental Health, 20: 400-403.

APPENDIX I

TYPICAL BIOCHEMICAL REACTION OF

Bacillus anthracis

Biochemical Tests	*Bacillus anthracis* Reaction
Gram staining	Gram positive
Motility	Non - motile
Capsular staining	Capsulated
Spores	Oval, do not swell cell
Simmon's citrate	Positive
Nitrate Reduction	Positive
Gelatin hydrolysis	Weakly positive
Urea - Christensens	Variable (+ or -)
Voges - Proskauer	Positive
Glucose	Acid production
Gas from glucose	Negative
Mannitol hydrolysis	Negative
Arabinose	Negative
Action on blood	Non-hemolytic
Growth at 25°C	Positive
Growth at 37°C	Positive
Growth at 42°C	Variable (Positive/Negative)

Source:
1. Cowan, S. T. and Steel, K. J., 1970
2. E. King, 1972
3. G. R. Carter, 1973.

APPENDIX II

GENERAL PRINCIPLES OF TANNAGE
VARIOUS TANNING METHODS

There are various tanning methods, these include:

1. POLYAROMATIC TANNAGE (VEGETABLE/SYNTHETIC)
   a. Pit tannage
   b. Accelerated tannage
   c. Rapid tannage (BASE RAPITAN Process)

2. MINERAL TANNAGE
   a. Chrome tannage
   b. Aluminium tannage
   c. Zirconium tannage

3. ALIPHATIC TANNAGE
   a. Aldehyde tannage
   b. Resin Tannage (Usually as pretannage or retannage)
   c. Oil tannage (for Chamois Leather)
   d. Immergan A tannage

4. COMBINATION TANNING METHODS:

The above tanning methods are used in various combinations with one another and in different

intensities to produce special leather properties. The dominating tanning method generally determines the final leather character. The retanning methods have gained significance, because they enable processing on an industrial scale and improve the use value of modern types of leather. For example, vegetable tans are often needed to chrome leather to improve the fullness or - firmness in planks or grain, whilst the bulk of the syntans are used in conjuction with other tannages to give a whiter leather or to speed up the tannage. "Semi-chrome" refers to complete vegetable tannage followed by retannage with chrome while "Chrome-retan" is a full chrome tannage followed by a vegetable or synthetic tannage.

TRADITION VEGETABLE TANNAGE

With the traditional method of tannage, which is subject to numerous variations and is evolving all the time, the skins of the animals slaughtered at dawn, are sprinkled with salt immediately and transfered to the tannery before mid-day.

They are resalted and left usually for about three days, after which they are soaked 5-8 hours in clean water, then soaked overnight in fresh water,

and on the following day given a third soaking in fresh water for one hour, all operations being performed in a pit without agitation of the water itself.

They are then limed, using either a slaked, poorly burnt lime or in other cases slaked shell lime. The pits, containing an approximate 2% suspension of the slaked lime, are plunged, and the skins are dropped in and pushed under the lime liquor one by one. They are drawn out of the liquor daily to drain, the liquor is again plunged and the skins returned one at a time to the liquor. At the seventh or eighth day, the hair is sufficiently loosened to permit unhairing by hand over a beam after which they are given a fresh lime liquor in which they remain for 4-6 days with daily hauling. They are fleshed by hand over the beam.

After washing with two changes of clean water, the skins are scudded by hand over a beam followed by several changes of water, in which they are trodden by bare-footed operatives. Usually an additional scudding is given between each successive soaking and treading operation. The ultimate result is clean, flaccid skins, sometimes with a tendency to roughening of the grain due to the hardness of the water.

Tanning is carried out in tubs containing 100 skins and old tan liquor, to which is added fresh 'Mimosa' bark (as liquid, solid or powder form) or 'Bagaruwa' extracts to produce a barkometer strength of 7-8. For the first three days, the skins are hauled out and put back twice daily, and on the fourth day are worked on the flesh side over a beam with a blunt unhairing knife to press the grain and get a good, square pattern. Thereafter they are replaced in the pits with the addition of more bark or extracts (both referred to as 'tannis') and the process is repeated several times to a total tanning time of 8-10 days. They are next transferred to a fresh liquor made up to a strength of 8-10 barkometer using "Marke" or "Bagaruwa" extracts but mostly, Bagaruwa's are used.

By the end of this time the goods are tanned through but are of poor colour containing iron and tannins' stains. They are then bleached by placing them for one day in a liquor made up of myrobalans and an extremely acid synthetic tan. This treatment removes stains and produces an excellent, yellowish colour. The skins are then horsed out, beamed on the flesh side to remove water, set out on a glass with a brass slicker,

sprinkled with sesame (or gingelly) oil on the grain side and laid grain to grain.

Thereafter the oil is massaged into the grain of the skins by hand, and the skins are hung to dry partially in the shade. When in a suitable semi-dry condition, they are again set out with brass slickers on a glass table to produce flatness, and finally are hung to dry. The last drying normally takes 24 hours, but during the worst periods of the raining season, the time can increase to three days. The skins are then staked over a steel stake, fluffed with slabs of very perous, overburnt brick, trimmed, sorted and baled.

LARGE SCALE TANNING PROCESSES

Generally, the pre-tanning processes are the same in all the different types of tannages.

In cases of raw hides or skins, they are cured by any of these processes;

a. Drying - gradual air drying by hanging or stretching out
b. Salting - Sprinkling with dry salt.
c. Brining - Suspension in brine and subsequent sprinkling with dry salt.

d. Dry-salting-combination of salting and drying or pre-drying first and then salting and final drying, or they are limed immediately.

As for dried hides or skins they are first soaked with fresh clean water in pits, paddles or drums at 28°C for three days and addition of chemicals like alkalis, acetic salt, soaking auxillaries and wetting agents depending on the types of hide or skin. Then followed by liming which could be done in any of these three ways.

a. Lime painting - using 130-150 litres lime paint per 1000 kg hides or skins.
b. Liming process
c. Eneymatic method

Deliming process follows and this is to remove chemically deposited and chemically bound lime during the liming process by conversion into readily soluble salts. Then bating which is further loosening and peptizing of the fibre texture of the skin and elimination of alkali swelling by the action of specific enzymes like pencreas proteinases (trypsin) mould fungus proteases and bacteria proteases. This is done at temperature ranging from 30-37°C.

Degreasing follows and this can be done either by wet degreasing of pelts and moist leathers at temperature ranging from 35-70°C depending on type of leather or dry greasing of leather pickling process of acidifying the pelts to a certain pH before chrome tannage. Thus, the astringency of the chrome tanning agents is reduced. Pickling is also used for preserving. This could also be done in any of these three ways.

a. Salt - containing pickle systems normally 8-10 Be with 1-3% acid.
b. Salt poor pickle systems, with the use of aromatic acids like Decaltal N powder and Picaltal Granules.
c. Short - pickling methods.

The various large scale tannage and retannage processes following depending on the type of tanning materials used. There are three different ones:

1. Polyaromatic tanning materials vegetable tannis and synthetic tanning agents.
2. Mineral tanning materials Polybases and Polyacids.
3. Atiphalic tanning materials Aldehyde tanning agents polycondesation and polymerzation compounds paraffin derivatives and fats.

The salts of many metals have the capacity to combine with skin protein, but only a few make a satisfactory leather. By far the commonest used is Chromium Sulphate in the chrome tanning process.

TYPICAL CHROME TANNAGE OF GLACE KID LEATHER
(FOR SHOE UPPERS)

1. Soak in vats: 500% water at 20°C.
   (% on raw skin weight) ½% sodium hypochlorite,
   for 24 hours.
2. Paint on flesh with: 10% sodium sulphide
   (% on volume of paint) 40% lime
   50% water
   Pile flat overnight
3. Unhair by hand or machine.
4. Lime in paddles with 500% water at 21°C
   (% on weight of raw skins) 10% hydrated lime
   1% sodium sulphide
   3-5% salt and
   paddle 5 minuted every 4 hours for 30 hours.
5. Neck - split not necessary
6. Flesh - machine
7. Scud
8. Delime in paddles in running water at 20°C.

(% on Scudded weight) for 30 minutes and then

500% water at 20°C

1% ammonium chloride for

60 minutes so that surfaces colourless to phenolphthalein (about pH 8.0)

9. Bate in paddles: 400% water at 38°C
(% on limed weight) 50% old bate liquor
1% Panereatic bate
run for 5-6 hours.

10. Chrome tannage (by two-bath or modified two-bath process).

Drum in about 100% cold water,

6% Sodium or Potassium dichromate.

4% hydrochloric acid (conc.)

For 3-6 hours, horsed up, covered to exclude light and drayghts and left overnight and then paddled in about 50% cold water.

20% hypochloric acid

10% hydrochloric acid.

After tanning using any desired tannage, the tanned hides and skins are brought to finishing through further processing which include neutralisation or de-acidification to remove the free acid present in mineral tanned leather or formed in the leather during storage by means of mild auxillaries without

damaging the leather fibre or the grain. Then application of emulsifiers and wetting agents in form of anionic products, cationic products and non-ionic products. The leather is then fatliquored, dyed, dried and put to finishing according to the finishing material, technique and effect.

Local vegetable tannage:

With the traditional method of tannage, which is subject to numerous variations and is evolving all the time, the skins of the animals, slaughtered at dawn, are sprinkled with salt immediately and transferred to the tannery before mid-day. They are resalted and left usually about three days, after which they are soaked.

Hide and skin defects:

Certain of the defects marring an otherwise beautiful leather have arisen in the life of the animal, others develop in the preparation of hides and skins and, although still outside the tanneries control, could with proper attention be avoided, yet others can be caused during tannery processing. It is important to be able to recognise the various types of defect so that those that can be are

avoided. The following list includes those defects commonly seen.

Damages caused during life:

1. Scars: When damages such as barbed wire and thorn scratches or horn rake are healing, the fibres grow densely packed together, and the scares are often hard and raised and without hair follicles. Damage may also be caused by brands, which are used to indicate ownership and are applied by hot iron.

2. Infestations: These are done mainly by warble flies, flies, ticks, sorcoptic and demodetic mange mites causing open or blind warbles, tick marks looking like pin-prick in the grain, or scare, roughened grain surface and shallow depression of tanned grain surface.

3. Infections: If ringworm, a fungus infection leaves no scar but if the animal is slaughtered while infected, the grain appears coarse at the site of the infection.

4. Cockle: Wool bearing sheep skins suffer from a peculiar seasonal disorder prior to shearing known as cockle.. This resembles small boil like, hard spots of varying size, which occur in rows at right angles to the backbone from the shoulder to the butt. It has been associated with mycotic dermatitis and with infestation of the animal by keds.

Damages caused after death:

5. Flaycuts and goughes: These affect the important corium fibres. In leather, they show through and thereby also spoil the grain.

6. Putrefaction: This is the result of bacterial growth which promptly starts once the animal is dead, especially in the epidermis, fibroblasts and muscle cells of the exposed flesh side of the flayed skin, unless it is properly cured. Often the first sign besides small of spoilage is hair slip, that is when the hide or skin is handled parches of hair and epidermis slip off the grain. And this is accompanied by a sensitive condition of the grain, so that the surface tends to rub away during normal processing. Slight rubbing gives "dull grain" and finishing is likely to be blotchy. Putrefaction also

causes the general structure of the skin to become rough and flabby. Bacteria can grow at any time before tanning when pH of the skin is about neutral, for instance when the skin is soaked, delimed or bated.

7. Veiness: Blood vessels can be seen on the flesh side as branching line. If owing to poor cure or old age, for example, the structure around them becomes loose, the skin is said to be veiny, and the branching pattern of the veins usually shows through on the grain side.

8. Coat damage: This is possible on hides and skins from tropical countries and is a common-trouble with ground - dried goods, that is by simply spreading them out on the ground, sometimes on a bed of twigs or stones. This may be dangerous owing to poor ventilation of the ground side and high temperature of the exposed side, plus contamination with dirts and soil bacteria. Anthrax bacteria, may be present on dried skins, in a spore form. Hides and skins are also susceptible to insect attack (beetles and maggots).

9. Red heat: This is a form of defect encountered in the process of curing. It gives deteriorated and devalued skins as a result of discolouration of the flesh side of the skin varying in intensity and shade. This is due to the pigment produced by chromogenic halophilic bacteria. (Martin, 1957 and Jovanovitis, 192[illegible]).

10. Salt stippen: This is also a defect of curing caused by fungus, otherwise known as "Fungus Stippen". It is attributed to the rupture of the grain tissue of the hide during the initial curing process as confirmed by histological investigations O'Flaherty, 1965). The damaged tissue is subsequently invaded by secondary bacteria.

11. Salt stains: Spectrographic and chemical analysis of stained areas in limed hides have shown that calcium phosphate is involved in producing salt stains. Calcium being derived from curing salt and phosphate from hide tissue break down. Predisposing factors in promoting salt stain formation have been attributed to prolonged storage, delayed curing and autolysis. (Pericaud, 1925 and Tancous, 1962).

12. White spots: Caused by micro-organisms like Leuconostoc Mesonteroides, Leuconostoc dextranicum Bacillus mesentericus fuxus and Akaligens Viscosus which survive the beam house processes to show up the defect on the leather due to their slimy fermentation on the hides suspended for drying.

Economic importance of leather:

In looking for a covering material for himself, his hut and food, the primitive man turned either to large leaves from plants or to the skins of the animals he killed. The latter were usually chosen for clothing as they were bigger, stronger and warmer, but had the defects of going damp, if left in a wet and especially warm climate, putrified and dried skins lost their flexibility and softness, liable to crack on bending and were quite unsuitable for clothing and other uses.

However, the modern man turned hides and skins to leather by tanning which do not putrefy even after drying and wetting and on drying tanned skins does not become a hard, brittle material, but remains flexible and workable.

With development, hides and skins whether in the raw form, as finished leather or as finished leather goods earn a majority percentage of foreign exchange to boost Nigeria's economy.

Economically, as reported in 1969, it was estimated that Nigeria exported 3,147 tons of hides worth approximately ₦621,000.00 giving average price per kilogram of ₦52.00 depending on the grade of the hide (Lloyd, 1973). This represents ₦2.80 per hide an average hide rejected hides on the other hand would sell about ₦1.00 or less locally, representing a loss of ₦1.80 per hide. (Lloyd, 1973, Bida, 1975).

APPENDIX III

DETAILED BIOCHEMICAL TESTS OF BACILLUS ANTHRACIS ISOLATES

1. Motility Test

This involved a semi-solid medium, using the craigie method. The medium in the inner tube was inoculated with the isolates and incubated at 37°C for 24 hours. Observations for growth through the sloppy agar was made after 24 hours.

2. Citrate utilization test

Solid Simmon's Citrate medium were inoculated with the isolates using a straight wire. The inoculated tubes were incubated at 37°C for 24 hours.

The isolates were citrate - positive, typical for Bacillus anthracis.

The colour of Simmon's Citrate medium is green before utilization and blue after utilization.

3. Urease Test

The solid medium was used, inoculated with isolates and incubated at 37°C for 24 hours. Observations showed that isolates urease negative, that is they don't posses urease which splits urea to form ammonia and changes the colour of the indicator in the medium from yellow to pink. Urease negative is typical of Bacillus anthracis.

4. Nitratase Test

This involves the reduction of nitrate to nitrate by nitratase enzyme.

The already prepared nitrate broth was inoculated with the isolates and incubated at 37°C for 24 hours. After this, few drops of 1N hydrochloric acid and 0.5ml of 0.2% solution of sulphanilamide and 0.5 ml of 0.170 N naphthylethlenediamine were added to the incubated broth. The solutions turned pink from light yellow. Signifying nitrate reduction to nitrite.

All the isolates were nitratase positive, typical of Bacillus anthracis.

5. Voges - Proskauer Reaction (VP Test)

This test for the formation of acetyl M methyl carbinol (acetroin) from glucose. This is oxidized by the reagent to diacetyl, which produces a red colour with guanidine residues in the media. For testing Bacillus species, 1% of sodium chloride was added to the MRVP medium (or Glucose phosphate broth).

The broth was inoculated with the isolates and incubated for 72 hours at 37°C. Then, the Banitt's method of using 3 ml of 5% alcoholic - naphthol solution and 3 ml of 40% potassium hydroxide were added to the incubated broth containing the growing isolates. A bright eosin red colour appeared in 5 minutes signifying a positive reaction.

All the isolates were positive for VP test, also typical of Bacillus anthracis.

6. Carbohydrate (Glucose) Utilization

This was a peptone broth base containing phenol-red as an indicator and an addition of 1% of the fermentable substance (Ammonium salt sugar). A Durham tube was included to detect gas production. The broth was inoculated with the isolates and incubated at 37°C for 24 hours.

The Bacillus anthracis isolates gave an acid production from the glucose but no gas in all the cases. This is also typical of Bacillus anthracis.

7. Arabinose

This is another fermentable substance for carbohydrate utilization.

The isolates were inoculated into the broth with Durham tubes. Incubated at 37°C for 24 hours.

The isolates did not utilize the arabinose, typical for anthrax bacilli.

8. Alcohol (Mannitol) utilization

This is another source of carbohydrate. The isolates were inoculated into the broth with Durham tubes. Incubated for 24 hours at 37°C, after which it was observed that they did not utilize the carbohydrate.

This is also typical of Bacillus anthracis.

9. Gelatin liquefaction

Bacillus anthracis is known to be a slow or non-gelatin liquefier.

The isolates were inoculated by stabbing the gelatin nutrient agar. They were incubated at 37°C because Bacillus species grow well in the liquid medium. Uninoculated control was included after seven days incubation, and both tubes were put in the refridgerator over-night. The control tubes olidified as well as the inoculated tubes. Most of them liquefied the gelatin between seven and nine days of inoculation that is slow liquefiers.

This is very typical of Bacillus anthracis.

Names of some special media used

1. Phenol - Ethyl alcohol Agar (PEA)

(Baltimore Biological Laboratory BBL):-
A division of Becton, Dickinson and Company. B-D; Cockysville, Maryland, U. S. A.).

Phenyl - ethyl alcohol agar is a selective medium designed by Brewer and Lilly for the isolation of Gram-positive organisms. It is useful where specimens are contaminated with Gram-negative organisms and in cases of mixed infections.

Formula in Grams per litre of distilled water

Tryplicase peptone	-	15.0 grams
Phytone peptone	-	5.0 g
Sodium chloride	-	5.0 g

2 - Phenyl ethyl alcohol - 2.5 g

Agar - 15.0 g

This was prepared with 7% of defibrinated sheep blood. The final pH = 7.3 ±.

2. Blood Agar Medium (BA)

This is nutrient agar or one of the commercial dehydrated Blood Agar Bases to which 7% of defibrinated sheep blood is added. It is used to detect haemolytic organisms and to encourage the growth of organisms that grow pooly or not all on nutrient agar. Since Bacillus anthracis is non-hemolytic on Blood Agar Medium, this helped in differentiating it from other Bacillus species because they are hemolylic on BA.

Formula in Grams/Litre of Distilled water

Lab - Lemco Powder (Oxoid L29)	- 10.0 g
Peptone (Oxoid L37)	- 10.0 g
Sodium Chloride	- 5.0 g
Agar	- 15.0 g

Plus 7% defilarinated sheep blood. The final pH is approximately 7.3.

3. Brain Heart Infusion (BHI) Broth

Formula in Grams per litre of distilled water

Calf brain (infusion solids)	-	12.5 g
Beef heart (infusion solids)	-	5.0 g
Protease peptone	-	10.0 g
Sodium chloride	-	5.0 g
Dextrose	-	2.0 g
Disodium phosphate anhydrous	-	2.5 g

Final pH - 7.4

4. Phosphate buffered saline (PBS)

This is a useful general diluent at pH 7.3

Formula in Grams per litre of distilled water

Sodium chloride	-	8.0 g
Potassium dihydrogen phosphate	-	0.34 g
Di-potassium hydrogen phosphate	-	1.21 g
Distilled water	-	1000 ml.

5. Yeast Extract Agar

Formula in Grams per litre of distilled water

Yeast extract	-	3.0 g
Peptone	-	5.0 g
Agar	-	15.0 g

APPENDIX IV

RECORDED HOURS OF DEATHS OF MICE INOCULATED WITH FIELD AND REFERENCE STRAINS OF *Bacillus anthracis*

Isolate number	Hours of death
RS 2	Between 18th and 24th hour
RS 3	Between 18th and 24th hour
RS51	No death of mice occurred
RS58	Between 18th and 24th hour
RS66	Between 18th and 24th hour
RS71	No death of mice occurred
RS72	Between 18th and 20th hour
RS89	Between 18th and 36th hour
RS90	Between 18th and 20th hour
RS102	At the 19th hour
LL 7	Between 18th and 20th hour
LL17	Between 18th and 24th hour
LL28	Between 18th and 36th hour
LL29	At the 24th hour
LL48	At the 24th hour
LL90	Between 18th and 22nd hour
LL101	Between 18th and 20th hour
LL103	Between 18th and 20th hour
SFL 4	At the 20th hour
SFL 24	At the 20th hour
SFL 56	Between 20th and 22nd hour
SFL 59	Between 20th and 22nd hour

SFL 87	At the 24th hour
SFL 101	Between 20th and 22nd hour
SFL 102	No death of mice occurred
Pathogenic reference strain	Between 18th and 24th hour
A virulent reference strain	No death of mice occurred

FOOTNOTE:

RS = Raw goat skin

LL = Locally-tanned leather

SFL = Scientifically tanned leather

APPENDIX V

BIOCHEMICAL REACTIONS OF NON-ANTHRAX BACILLI ISOLATED FROM FRESH FLAYED GOAT SKINS

Sample Number	Gram-Reaction	Capsular Reaction	Motility	Citrate	Urea	Nitrate	Voges Proskauer	Glucose	Arabinose	Mannitol	Gelatin Liquefation
RS 4	Gm +ve long rods	C	+	+	+	+	+	A	A	-	R
RS 5	"	C	+	+	+	+	+	A	A	-	R
RS 6	"	C	+	+	+	+	+	A	-	-	R
RS 7	"	C	+	+	+	-	+	A	-	-	R
RS 9	"	C3	+	+	+	+	+	A	-	-	R
RS11	"	C	+	+	+	+	+	A	A	-	R
RS14	"	C	+	+	+	+	+	A	-	-	R
RS16	"	CS	+	+	+	+	+	A	-	-	R
RS24	"	C3	+	+	+	-	+	A	-	-	R
RS27	"	C	+	+	+	-	+	A	-	-	R
RS28	"	C	+	+	+	+	+	A	-	-	R
RS37	"	C3	+	+	+	+	+	A	-	-	R
RS44	"	C	+	+	+	+	+	A	-	-	R

APPENDIX V (Cont.)

RS 45	Gm +ve long rods	C	+	+	+	+	+	A	-	-	R
RS 46	"	C	+	+	+	+	+	A	-	A	R
RS 47	"	CS	+	+	+	+	+	A	-	-	R
RS 50	"	C	+	+	+	+	+	A	-	-	R
RS 52	"	C	+	+	+	+	+	A	-	-	R
RS 53	"	C	+	+	+	-	+	A	-	-	R
RS 55	"	CS	+	+	+	-	+	A	-	-	R
RS 56	"	C	+	+	+	-	+	A	-	-	R
RS 57	"	C	+	+	+	-	+	A	-	-	R
RS 61	"	CS	+	+	+	+	+	A	-	-	R
RS 62	"	C	+	+	+	+	+	A	-	-	R
RS 63	"	C	+	+	+	-	+	A	-	-	R
RS 65	"	C	+	+	+	+	+	A	-	-	R
RS 69	"	C	+	+	+	+	+	A	-	-	R
RS 70	"	C	+	+	+	+	+	A	A	-	R
RS 74	"	C	+	+	-	+	+	A	-	-	R
RS 76	"	CS	+	+	+	+	+	A	-	-	R

APPENDIX V (Cont.)

RS 77	Gm +ve long rod	C	+	+	-	+	+	A	-	-	R
RS 79	"	C	+	+	+	-	+	A	-	-	R
RS 80	"	C	+	+	-	+	+	A	-	-	R
RS 84	"	C	+	+	-	+	+	A	A	-	R
RS 86	"	CS	+	+	+	+	+	A	-	-	R
RS 87	"	C	+	+	+	-	+	A	-	-	R
RS 92	"	C	+	+	+	+	+	A	-	-	R
RS 93	"	CS	+	+	+	+	+	A	-	-	R
RS 97	"	C	+	+	+	+	+	A	-	-	R
RS101	"	C	+	+	+	+	+	A	-	-	R

Foot Note: RS = Raw skin sample
Gm+ve = Gram positive long rods
C = Capsulated but no spores
CS = Capsulated with spores
+ = Positive reaction
- = Negative reaction
A = Acid production
R = Rapid gelatin liquefaction (between 3-4days)

APPENDIX VI

BIOCHEMICAL REACTION OF NON-ANTHRAX BACILLI ISOLATED FROM BOTH LOCALLY-TANNED AND SCIENTIFICALLY-TANNED GOAT LEATHER

Sample number	Gram Reaction	Capsular Reaction	Motility	Citrate	Urea	Nitrate	Voges Proskauer	Glucose	Arabinose	Mannitol	Gelatin Liquefaction
LL 23	Gm+ve Long rods	CS	-	+	-	+	+	A	A	A	R
LL 30	"	CS	-	+	-	+	+	A	A	-	R
LL 54	"	CS	-	+	-	+	+	A	-	A	R
LL102	"	CS	-	+	-	+	+	A	A	A	R
SFL 74	"	CS	+	+	-	+	+	A	A	A	R
SFL 89	"	CS	-	+	-	+	+	A	-	-	R
SFL103	"	CS	+	+	-	+	+	A	A	A	R

Foot note:
LL = Locally-tanned goat leather
SFL = Scientifically tanned goat leather
Gm+ve = Gram positive long rods
CS = Capsulated with spores
+ = Positive reaction
- = Negative reaction
A = Acid production
R = Rapid gelatin liquefaction (Between 1-4 days).