ISOLATION AND TAXONOMIC ANALYSIS OF BACILLUS SPECIES IN ANTHRAX NEGATIVE CASES AT CENTRAL VETERINARY LABORATORY, NAMIBIA

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Abstract

The emergence of anthrax-like infections caused by *Bacillus* spp. other than *Bacillus anthracis* is quickly becoming a topical issue. This brought more concern to the scientific community, guardians of public health, and veterinarians around the world. Some bacillus species that cause anthrax-like infections harbor plasmids similar to those in *Bacillus anthracis* that encode for toxin genes while in some cases anthrax-like etiologic agents have not been investigated for virulence genes. In some instances, however, anthrax-like etiologic agents harbor virulence genes different from those of *B. anthracis* yet still cause anthrax-like infections. The present study aimed to isolate bacillus species from anthrax-negative samples and analyses the possibility of isolates to cause anthrax-like infections. The samples used were obtained from the Central Veterinary Laboratory (CVL) in Windhoek, Namibia. The samples analyzed in the study were submitted to CVL over the period of May 2019 to March 2020 for *B. anthracis* test, but they tested negative. Twenty-three (23) samples were obtained and analyzed for the presence of *Bacillus* spp. by culture methods. This was followed by DNA extraction and then amplification of the 16S rDNA gene using universal 16S bacterial primer sets 27F and 1492R. From the 23 original samples, 13 different *Bacillus* spp. were identified by BLAST search on NCBI. The identified species were *Bacillus australimaris, Bacillus pumilus, Bacillus subtilis, Bacillus simplex, Bacillus megaterium, Bacillus licheniformis, Bacillus albus, Bacillus aryabhattai, Bacillus acanthi, Bacillus thuringiensis, Bacillus cereus, Bacillus zhangzhouensis* and *Bacillus safensis*. Due to COVID-19 related complication, the isolated bacillus species could not be tested for the presence of virulence genes. However, statistical determinations were carried out to determine if there were correlations between

the isolated strains and the symptoms presented by the animals from which the samples were obtained. The chi square value is 0.104 which is greater than the significant value (p=0.05). Of the isolated bacillus species *B. cereus, B. pimulus,* and *B. megaterium* where once isolated from anthrax-like infection. This study isolated bacillus species from anthrax negative samples that may not be responsible for anthrax-like infections based on the statistical analysis. However, screening of virulence genes in further studies is recommendable to prove this conclusion.

Keywords: Anthrax-like infections, anthrax-like isolates, bacillus species

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List of Abbreviations and/or Acronyms

AFLP	Amplified fragment length polymorphism
BLAST	Basic Local Alignment Search Tool
BLASTn	Basic Local Alignment Search Tool for nucleotide
Bv	biovars
CAM	Cameroon
CI	Côte d'Ivoire,
CVL	Central Veterinary Laboratory.
DNA	Deoxyribonucleic acid.
dNTPs	deoxynucleoside triphosphates
DRC	Democratic Republic of Congo
EF	edema factor
ISO	International Standard Organization
kbp	kilobase pair
LF	lethal factor
MEGA	Molecular Evolution Genomic Analysis
MgCl	Magnesium chloride
min	minutes
MLST	Multilocus sequence typing

MLVA	Multiple loci VNTR analysis
NCBI	National center for Biotechnology information
NSG	no symptoms given
ORFs	Open reading frames
p	significant value
PA	protective antigen
PCR	Polymerase chain reaction
RCA	Central African Republic
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
sec	Seconds
spp.	Species
SPSS	Statistical Package for the Social Sciences
TAE	Tris-acetate-EDTA

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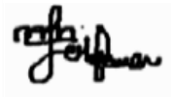
Dedication

This thesis is dedicated to my Sister **Rachel NT Nghifewa** and all who waited with me during my studies. May the Almighty God increase their portions!

Declarations

I, Mateus Ndinovamati Nghifewa, hereby declare that this study is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institution.

Mateus Ndinovamati Nghifewa		02/07/2021
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1. CHAPTER ONE: INTRODUCTION

1.1. Background of the study

Bacteria of the genus *Bacillus* are classified into the family *Bacillaceae*. It contains endospore forming Gram-positive rod-shaped bacteria. The genus exhibits a wide range of physiologic properties enabling its species to be ubiquitous in nature (Priest, 2008). For instance, spores are resistant to many environmental conditions such as radiations, temperatures, disinfectants and chemical sterilants that are lethal to vegetative cells (Blackburn and McClure, 2009). There is a *Bacillus cereus* group that house the *B. anthracis, B. cereus, B. thuringiensis, B. weihenstephanensis, B. pseudomycoides* and *B. mycoides* (Marston *et al.*, 2006; Klee *et al.*, 2010). The species *Bacillus anthracis* is a well-known etiological agent of anthrax (Öncü *et al.*, 2003; Cloeckaert *et al.*, 2020; Akula *et al.*, 2005; Amesh, 2011; Hoffmaster *et al.*, 2004). Anthrax is an acute lethal zoonotic disease in which the bacteria can be present in blood and it is also associated with toxemia (Brézillon *et al.*, 2015).. Specifically anthrax epidemics lead to the mortality of wildlife(Siamudaala, 2005; Öncü *et al.*, 2003; Cossaboom *et al.*, 2019; Bhattacharya *et al.*, 2013).

Virulent forms of *B. anthracis* harbor two plasmids: pXO1 (181.6 kb) and pXO2 (93.5 kb), which are virulent plasmids (Antonation *et al.*, 2016). The virulence genes found on plasmids are responsible for toxin and capsule production in the pathogenesis of *Bacillus anthracis* (Priest, 2008). The encoded toxins are protective antigen, edema factor and lethal factor (PA, EF and LF respectively) (Patrick *et al.*, 2009). The virulence genes are known as *pagA, lef,* and *cya* encoding for PA, EF and LF respectively (Scarff *et al.*, 2016; Klee *et al.*, 2010). However, the existence of a distinct clade of *Bacillus cereus* isolates

causing anthrax-like disease in a range of wild and domestic mammals and humans in tropical Africa and other parts of the world has also been reported (Baldwin, 2020; Duncan and Smith, 2011; Tena *et al.*, 2007). In some cases, the ability of those bacillus species to cause anthrax-like disease is also due to their two plasmid-borne virulence genes (Brézillon *et al.*, 2015).

These anthrax-like etiologic agents have been known since 1965 (Amesh, 2011). However, the existence of these anthrax-like etiologic agents has been undermined as their isolation, other than that of *B. anthracis*, is normally considered as environmental contamination (Amesh, 2011). In what could signal a change of the tide, *Bacillus cereus* were recently isolated from anthrax suspected cases and proven to be virulent, rather than mere contaminants (Klee *et al.*, 2010). These *B. cereus* strains have been proven to contain plasmids with virulence genes which are highly similar to those genes found in pXO1 and pXO2 of *B. anthracis* (Amesh, 2011; Antonation *et al.*, 2016; Hoffmaster *et al.*, 2004; Klee *et al.*, 2010; Wright *et al.*, 2011).

Besides the anthrax-like isolates, there has been isolation of other bacillus species from anthrax-like disease cases (Cloeckaert *et al.*, 2020; Hoffmaster *et al.*, 2006; Hoffmaster *et al.*, 2004; Brézillon *et al.*, 2015; Wright *et al.*, 2011). Together, these findings draw more research focus towards the role played by non- *Bacillus anthrancis* spp. in human and animal's health. This argument is further bolstered by the findings of (Han *et al.*, 2006) who proved that some bacillus species namely *Bacillus cereus* E33L and *Bacilluss thurigeniss* 97-27 possess virulence genes, including five genes that are homologous to virulence genes encoded by *Listeria monocytogenes*, as well as antibiotic resistance operons that may increase their virulence. *Bacillus cereus* strains, in particular, are noted

for their ability to cause anthrax-like infections (Cloeckaert *et al.*, 2020). This, and the fact that *B. cereus* are highly ubiquitous in the environment makes them priority organisms with potential to pose significant health risks to both people and animals (Hoffmaster *et al.*, 2006). While this organism has been isolated from different geographic and ecological areas (Baldwin, 2020; Klee *et al.*, 2010; Hoffmaster *et al.*, 2004), there is a dearth of information on the prevalence and public health significance of environmental *Bacillus cereus* in Namibia. Of what is currently known, (Han *et al.*, 2006) report the isolation of *B. cereus* E33L from soil samples collected around a zebra carcass that was believed to have died of anthrax in Etosha National Park, Namibia. While this particular *B. cereus* strain (E33L) did not possess either of the virulence genes or plasmids of *B. anthracis*, more widespread studies targeting etiology in anthrax-like infections will provide useful information in Namibia. The aim of the present study is to isolate and analyze bacillus species from anthrax negative cases to identify possible anthrax-like etiological agents.

1.2. Statement of the problem

There have been reported incidences of suspected anthrax cases in Namibia (Anon, 2019b) and anthrax-like infections around the world (Wright *et al.*, 2011; Baldwin, 2020; Han *et al.*, 2006). While in some instances anthrax suspected diseases are not confirmed for *B. anthracis* by culture methods, the real etiological agent behind these cases of animal mortality remains unknown and are not explored (Cossaboom *et al.*, 2019; Amesh, 2011). Recent studies report the emergence of bacillus species causing anthrax-like disease in sub-Saharan Africa (Marston *et al.*, 2016) and other different parts of the world (Cloeckaert *et al.*, 2020; Baldwin, 2020). The strains were isolated from infections that presented symptoms similar to those presented by *Bacillus anthracis* infections

(Antonation *et al.*, 2016; Marston *et al.*, 2016; Hoffmaster *et al.*, 2006; Hoffmaster *et al.*, 2004). This study, therefore, aimed to isolate and carry out molecular characterization of non-*anthracis Bacillus* spp. from anthrax suspected samples that returned negative *B. anthracis* results at the Central Veterinary Laboratory (CVL) in Windhoek, Namibia.

1.3. Objectives

The specific objectives of this study were:

a) To collect sample information of anthrax suspected but negative samples from CVL and isolate *Bacillus* spp. by culture method.
b) To extract bacterial genomic DNA from the isolated *Bacillus* spp. from anthrax suspected but negative samples from CVL for sequencing analysis.
c) To perform taxonomic identification of isolated *Bacillus* spp. and analyze the relationship between the *Bacillus* spp. with symptoms presented.

1.4. Hypothesis of the study

This study was premised on the hypothesis that:

Suspected anthrax cases that tested negative for *B. anthracis* will present *Bacillus* spp. that will be related to the symptoms presented.

1.5. Significance of the study

The identification of other bacillus species from suspected anthrax cases that return negative tests for anthrax will shed light on the etiological agents of anthrax-like disease in Namibia. The information will also guide future researcher on anthrax-like diseases in Namibia as it will add on the existing information of anthrax-like diseases.

1.6. Limitation of the study

The current study only focused on disease case samples submitted at CVL in the period of 2019-2020 and no considerations were given to other laboratories that test for anthrax in Namibia. Due to the limitations of materials provided, the study limited the number of sample to less than 25 samples. The study did not include samples such as borne and meat submitted for food hygiene, but only focused on the clinical samples or disease cases submitted for diagnosis of *Bacillus anthracis*. The study only considered animal samples submitted at CVL. There was no positive control used in this study because the isolation of bacillus species did not target a specific bacillus species henceforth the microbial features of bacillus species were followed in order to isolate bacillus species.

1.7. Delimitation of the study

The bacillus species that were isolated may not be related to other anthrax-like isolates that have been isolated in other studies due to different locations and genetic diversity of bacteria.

2. CHAPTER TWO: LITERATURE REVIEW

2.1. Anthrax: A description of the etiologic agent and the clinical manifestation of anthrax disease

Anthrax is a zoonotic disease whose etiological agent is *Bacillus anthracis* (Scarff *et al.*, 2016). *Bacillus anthracis* is a spore-forming, Gram positive, rod shaped bacterium. The diseases caused by this bacteria have been a key in shaping the history of zoonosis (Stevenson, 2020). Scholars refer to the fifth plaque (15 century BC) that is reported to have killed Egyptian cattle (as recorded in the Book of Genesis) to have been caused by anthrax (Serkan *et al.*, 2013). Robert Koch was the first to isolate *B. anthracis* by culturing methods in the laboratory (Blevins and Bronze, 2010; Stevenson, 2020). Robert Koch was German physician and one of the founders of bacteriology. His work was based on the germ theory by Louis Pasteur. Robert Koch first isolated *B. anthracis* in 1877 and from the same research he summarized four criteria which are now called Koch's postulates (Stevenson, 2020).

Bacillus anthracis is a large, square-ended, facultatively anaerobic or aerobic Gram-positive bacterium (Blevins and Bronze, 2010). It is non-hemolytic on blood agar, non-motile, and can be lysed by gamma-bacteriophage. Its cells appear in chains, which are normally not encapsulated when grown in culture (Serkan, Selcen and Serhan, 2013). Sporulation occurs *in vitro* and is not common *in vivo* except if exposed to air then the bacteria can form spores. It grows well within 18 to 24 hours at 35 °C (Serkan *et al.*, 2013; Klee *et al.*, 2006). Its colonies are gray or white in color, irregular and flat, opaque, the diameter is in the range of 4 to 5 mm, with a slightly undulate margin (Serkan *et al.*, 2013).

The bacterium is considered as highly monophyletic clade, and isolates can be differentiated with variable number of tandem repeats (VNTRs) and single nucleotide polymorphism (SNPs) identifications (Klee *et al.*, 2010). Phenotypic approaches to isolate this bacterium are useful in laboratory diagnosis. A summarized approach to their identification is shown in **Table 2.1**. This approach differentiates *B. anthracis* from other bacillus species, and it is therefore necessary for laboratory operational procedures, specifically the gamma phage test as it is widely used (Serkan *et al.*, 2013).

Table 2.1: The phenotypic and biochemical characteristics of *Bacillus anthracis*.

Species	***Bacillus anthracis***
Motility	–
Hemolysis on blood agar	–
Capsule	+
Catalase Production	+
Para sporal Bodies	–
Lipid globules in protoplasm	+
Lecithovitellin reaction	+
Citrate utilisation	V
Anaerobic growth	+
V-P Reaction	+
pH in V-P medium<6.0	+
Growth in 7% NaCl	+
Acid from AS glucose	+
Acid + gas from AS glucose	–
Nitrate reduction	+
Casein hydrolysis	+
Starch hydrolysis	+

Notes: V-P, Voges-Proskauer, AS, Ammonium Salt, V-Variable.

Molecular studies show that the virulence genes of *B. anthracis* are plasmid-borne, and are responsible for encoding three protein toxins and capsule biosynthesis (Klee *et al.*, 2010). The three toxins work in synergy for them to be effective in initiating the infection. To repeat, these toxins are known as protective antigen, edema factor and lethal factor (PA, EF and LF respectively) (Patrick *et al.*, 2009). The virulence genes on plasmid that encode for toxins are *pagA, lef,* and *cya* encoding for PA, EF and LF respectively (Scarff

et al., 2016; Klee *et al.*, 2010). *B. anthracis* cause edema and cell death by these tripartite toxin based on their two plasmids pXO1 and pXO2 which encode the tripartite toxin and the poly-c-D-glutamic acid capsule, respectively (Klee *et al.*, 2010).

Animals become infected when they ingest spores by grazing on contaminated land or eating contaminated food. The disease may also be spread from animal to animal by via means such as biting flies, non-biting flies and by vultures (Akula *et al.*, 2005). The disease affects a wide range of wild animals such as kudu *(Tragelaphus strepsiceros)*, impala *(Aepyceros melampus)*, buffalo *(Syncerus caffer)* and zebra *(Equus spp.)* (Ngetich, 2019). Infected animals bleed from orifices (Ghenghesh *et al.*, 2014) and animals may present signs such as staggering, excitation/somnolence, spams, colic and swelling around the neck, abdomen and chest, convulsions and recumbence. Animals are normally found dead near water points or grazing areas where previous cases may have been reported. Incomplete rigor mortis, and rapid bloating can be noted and ulcers may also be noted in the oral cavity of a dying animal (Ngetich, 2019). There are three main forms of anthrax, it is therefore best to provide a description on forms of anthrax prior to explaining anthrax-like disease.

2.1.1. Inhalation anthrax

This form of anthrax is a result of inhalation of *B. anthracis* spores (Lins *et al.*, 2019). Inhalation anthrax is preferable called anthrax pneumonia, once the spores reach the alveolar they are ingested by the macrophages. Macrophages transport the spores to lymphatics then to mediastinal lymph nodes, resulting in the germination of spores. The disease develops within a week from the time of exposure but, this can also be long as 2 months, therefore the germination of spores can take up to 60 days (Orton and Wartz,

2001; Anon, n.d.; Lins *et al.*, 2019). Hemorrhagic meningitis, with nuchal rigidity and abtundation can also be noticed. Prognosis is poor in this form even with intensive antibiotic therapy. After the development of the disease death can occurs within 36 hours and without antibiotic treatment the mortality rate is 100% (Ghenghesh *et al.*, 2014; Orton and Wartz, 2001).

2.1.2. Cutaneous anthrax.

Cutaneous anthrax is the infection form of anthrax that affects the skin (Anon, n.d.). Cutaneous anthrax is normally observed in humans usually those who had contact with animals or animal products (Bhattacharya *et al.*, 2013) such wools and it usually affects the arms, face and neck. This form of anthrax is a result of the introduction of *B. anthracis* spores in skins breaks or abrasions hence initiating spore germination. The germination of spores in the abrasions of skin lead to the formation of papule in few days. The papule matures into a vesicle with fluids from which the etiologic agent can be recovered. The fluid in the vesicle is normally bluish dark in color (Ghenghesh *et al.*, 2014; Orton and Wartz, 2001; Tena *et al.*, 2007).

2.1.3. Gastrointestinal anthrax.

Gastrointestinal anthrax has the same principal as cutaneous anthrax (Bhattacharya *et al.*, 2013; Akula *et al.*, 2005), however this occurs as a result of the introduction or entry of spores through the mucosa. It is the intestinal form of anthrax that occurs as a result of ingestion of the spores. In humans, this can result from ingesting spore via undercooked spores in contaminated meat (Nakanwagi Id *et al.*, n.d.). Mortality rates for this type of anthrax are above 50% due to difficulty in early diagnosing (Ghenghesh *et al.*, 2014; Orton and Wartz, 2001).

2.1.4. Other form of anthrax.

There are two more forms of anthrax which are; anthrax meningitis and oral-oropharyngeal anthrax. Anthrax meningitis is a result of bacteremia that occurs after any other form of anthrax, and this form is almost always fatal even when intensive antibiotic therapy is involved (Ghenghesh *et al.*, 2014; Orton and Wartz, 2001). Oral-oropharyngeal anthrax is an unusual form that occurs without any sign of other forms and it normally occurs in humans. Oral-oropharyngeal anthrax is a fatal form that is characterized by mucosal lesion in oropharynx and/or oral cavity (Ghenghesh *et al.*, 2014; Orton and Wartz, 2001).

2.2. Anthrax-like disease or anthrax-like infections

The term anthrax-like disease or anthrax-like infections in this study refers to infections that mimic anthrax disease, and are caused by *Bacillus* spp. other than *B. anthracis*. The terms anthrax-like diseases and anthrax-like infections will be used interchangeably in this study. Anthrax-like infections have been reported in different parts of the world (Cloeckaert *et al.*, 2020) affecting both humans and animals (Hoffmaster *et al.*, 2004; Pilo *et al.*, 2011; Klee *et al.*, 2010). Anthrax-like infections are caused by bacillus species other than *B. anthracis*, with the species *Bacillus cereus* causing the most anthrax-like infections (Cloeckaert *et al.*, 2020). However, anthrax-like infections have not been limited to *B. cereus*. In most of the cases of anthrax-like diseases, it was proven that etiologic agents of anthrax-like disease contain virulence genes similar to those found encoded in two plasmids of *B. anthracis* (Marston *et al.*, 2016). Anthrax-like infections may mislead diagnosis because they present symptoms similar to anthrax yet the causative agents are not identified as *B. anthracis* (Tena *et al.*, 2007; Klee *et al.*, 2006; Klee *et al.*, 2010;

Marston *et al.*, 2016; Wright *et al.*, 2011; Pilo *et al.*, 2011; Antonation *et al.*, 2016; Öncü *et al.*, 2003).

However, there is a paucity of information on the bacillus species that cause anthrax-like infections. Many identified anthrax-like isolates were *B. cereus* strains that harbor virulence genes with different similarities (Cloeckaert *et al.*, 2020). Furthermore, *Bacillus pumilus* and *Bacillus megaterium* have been isolated from cutaneous anthrax-like eschar though they have not been explored if they harbor virulence genes of *B. anthracis* (Emmert, 2011; Duncan and Smith, 2011; Tena *et al.*, 2007). Therefore, exploration of anthrax-like disease may provide more information. Due to the paucity of information on anthrax-like infections, when other bacillus species are isolated during the diagnosis of anthrax they are discarded as environmental contaminants in some cases (Amesh, 2011).

2.3. *Bacillus anthracis* virulence genes associated with *Bacillus cereus* strains causing anthrax-like disease

There have been anthrax-suspected cases that tested negative for *Bacillus anthracis*. These cases have been investigated in both humans and animals. Examples include the inhalation anthrax that was suspected from human (Hoffmaster *et al.*, 2004) and cutaneous anthrax-like infection have been observed (Saikia *et al.*, 2019). The etiological agents for the isolates involved in anthrax-like infections in some cases contain virulence gene encoded in pXO1 and pXO2 plasmid of *B. anthracis* (Wright *et al.*, 2011; Klee *et al.*, 2010). Therefore, there are other isolates from anthrax suspected cases that were not *Bacillus anthracis*, instead they were *Bacillus cereus* (Baldwin, 2020) and they are responsible for causing anthrax-like infections due to the virulence gene they encode. Examples include

B. cereus E33L and *Bacillus cereus* Elc2 (Han *et al.*, 2006; Wright *et al.*, 2011) and others as can be seen on **Table 2.2**.

The pXO1 and pXO2 plasmids of *Bacillus anthracis* (Dale and Park, 2010; Klee *et al.*, 2010) as discussed under **section 2.1**, carry virulence genes that are responsible for the pathogenesis of *B. anthracis* in anthrax. Anthrax-like isolates have genes that are homologous to *pagA, lef,* and *cya*, and encode for PA, EF and LF respectively (Hoffmaster *et al.*, 2004; Marston *et al.*, 2016; Patrick *et al.*, 2009; Cloeckaert *et al.*, 2020) which have been detected in *Bacillus cereus* strains (Hoffmaster *et al.*, 2004). These genes are suspected to be responsible for anthrax-like diseases because they were detected in isolates of *B. cereus* from anthrax-like disease cases. Therefore, researchers have concluded that *B. cereus* can become virulent and cause anthrax-like disease when they possess and express these virulence genes (Dale and Park, 2010; Marston *et al.*, 2016).

Bacillus cereus species isolated from anthrax-suspected samples have been long referred to as contaminants (Amesh, 2011). Specifically, the isolation of *B. cereus* from anthrax-suspected cases that tested negative for *B. anthracis* has long been considered as an environmental contamination (Amesh, 2011). In most cases, these isolates end up being discarded instead of their presence being further investigated. Therefore, the possibility of isolates that may possess pXO1 and pXO2 or specifically these plasmids homologous virulence genes may have been missed often. Unexpectedly, research has also shown that genes that have been considered to be found only in *B. anthracis* are in fact more widely distributed (Hoffmaster *et al.*, 2004) and that *B. anthracis* pXO1 and pXO2 homologous sequences have been detected in other bacillus species (Hoffmaster *et al.*, 2004; Antonation *et al.*, 2016; Klee *et al.*, 2006; Marston *et al.*, 2016). Before a description on

some anthrax-like isolates is provided, the plasmids that harbor virulence genes or encodes for virulence attributes will be explained.

2.3.1. Plasmids found in bacillus isolates that may encode for virulence genes

Bacillus cereus species have been implicated in anthrax-like disease as it is the most isolated and explored species in regard to anthrax-like infections. Besides *B. anthracis*, it is also the species from which plasmids encoding virulence genes have been frequently isolated. These virulence genes are responsible for coding the biosynthesis of toxins (Hoffmaster *et al.*, 2004; Wilson *et al.*, 2011). These plasmids have high homology with pXO1 and pXO2, and carry with them the backbone of virulence genes in different percentage similarities amongst themselves and also when compared to the plasmids of *Bacillus anthracis* (Hoffmaster *et al.*, 2006; Klee *et al.*, 2006).

In most cases the plasmids from anthrax-like isolates that have high homology with pXO1 and pXO2 are named differently to indicate that they are not the normal *B. anthracis* plasmids (**Table 2.2**). For example, *B. cereus* G9241 (Hoffmaster *et al.*, 2006) also cause anthrax-like symptoms and have plasmids encoded factors. It harbors pBCXO1 which is highly similar to pXO1 of *B. anthracis*. In addition, some plasmids that encode for virulence attributes in anthrax-like isolates are not homologous to those found in *B. anthracis*. For example, *B. cereus* G9241 harbors pBC210 plasmid that encode a polysaccharide capsule (Klee *et al.*, 2010; Hoffmaster *et al.*, 2004). *B. cereus* 03BB102 harbors a plasmids named p03BB102_179. Astonishingly, this plasmid contain together the anthrax toxin and capsule biosynthesis genes (Klee *et al.*, 2010).

2.4. Bacillus species isolated from anthrax-like infections with virulence genes of *B. anthracis*

The presence of *Bacillus anthracis* virulence genes in other bacillus species is of concern since it is expected that these virulence genes can only be found in *B. anthracis* because it is the causative agent of anthrax (Blevins and Bronze, 2010; Öncü *et al.*, 2003). Due to the presence pXO1 and pXO2 encoded virulence genes in other bacillus species the species isolated from anthrax-like infections with virulence genes are therefore referred to as anthrax-like isolates (Leendertz *et al.*, 2006; Duncan and Smith, 2011). In this study, the term anthrax-like isolates refers to *Bacillus spp.* responsible for anthrax-like infections. In most cases, isolated *Bacillus spp*. are not screened for pXO1 and pXO2 virulence genes (Amesh, 2011).

However, *Bacillus cereus* infections are life threatening if the bacterium possesses virulence plasmids, thus informing the argument not to disregard non-*anthracis* bacillus species isolated in cases when anthrax symptoms are presented, such as in cases of bacteremia, pneumonia and sepsis (Amesh, 2011). In some instances, *B. cereus* plasmids-borne virulence genes have more than 70% similarity with those borne on *B. anthracis* plasmids (Wright *et al.*, 2011). However, research shows that *B. cereus* with virulence genes are not modified in any way and are not *B. anthracis* mutants. They differ from *B. anthracis* phenotypically and in their biochemical reactions (**Table 2.1**) because they are motile, test negative for the phage test, and some isolates are resistant to penicillin G (Klee *et al.*, 2010; Wright *et al.*, 2011).

Table 2.2: Some of the bacillus species isolated from anthrax-like disease.

Isolate name	Place of isolation	Virulence plasmids	Site or Organism of isolation	Accesses ion Number on GeneBank	References
***Bacillus* cereus biovar anthracis CA**	CI, CAM, RCA DRC	pBCXO1 pBCXO2	Elephant Gorilla Goat	SAMN03610233 to SAMN03610237	(Antonation *et al.*, 2016)
***B. cereus* biovar anthracis strain CI**	Ivory coast	pBCXO1, pBCXO2	Chimpanzee ‘‘Leo’’	CP001746–CP001749	(Klee *et al.*, 2010; Klee *et al.*, 2006)
***B. cereus* JF3964**	Koza, Cameroon	pBCXO1 pBCXO2	Bovine	FR872834-FR872889.	(Pilo *et al.*, 2011)
***Bacillus cereus* Elc2**	Rural area of Texas	pBCXO1	Human Welder	Not known	(Wright *et al.*, 2011)
***B. cereus* 03BB108**	Texas and Louisian	NP	Worksite of welder	Not known	(Hoffmaster *et al.*, 2006)
***B. cereus* 03BB102**	Texas	p03BB102_179	Welder	Not known	(Hoffmaster *et al.*, 2006)
B. cereus 03BB87	Texas	pBC218	Muller operator	Not known	(Hoffmaster *et al.*, 2006)
***B. cereus* strain G9241**	Louisiana	pBCXO1 pBC218	Human	AY425946	(Hoffmaster *et al.*, 2004)
***B. cereus* isolate, BcFL2013**	Florida	pBCXO1	Human	JHQN01000000	(Marston *et al.*, 2016)
***B. cereus* E33L**	Namibia Etosha national park	NP	Soil around dead Zebra	NC_006274	(Han *et al.*, 2006)
***B. thuringiensis* serovar konkukian strain 97-27**	Paris, France	NP	Human tissue necrosis	NC_005957	(Han *et al.*, 2006; Hernandez *et al.*, 1998)

Note: CI- Côte d'Ivoire, CAM- Cameroon, RCA- Central African Republic elephant (A363/2), RCA gorilla (A-364/1) and DRC- Democratic Republic of Congo goat (14-0024-1). NP- no virulence plasmid homology to *B. anthracis*. In addition, the *B. thuringiensis* serovar konkukian strain 97-27and *B. cereus* E33L were isolated from disease cases, they show to be closely related to those strains with anthrax-virulence genes although these two strains do not encode the virulence genes.

The description on some few anthrax-like isolates will be described below. This will give a brief idea on how most of the anthrax like isolates have been explored and their differences based on the genes responsible for anthrax-like infections. This provide an general expectations to the researcher if one have to investigate anthrax-like infections as it has been done previously (Emmert, 2011; Wright *et al.*, 2011; Baldwin, 2020; Ma *et al.*, 2018).

2.4.1. *Bacillus cereus* biovar anthracis strain CI and *Bacillus cereus* biovar anthracis strain CA

Bacillus cereus biovar anthracis has been isolated from domestic and wild animals (Antonation *et al.*, 2016). These strains named *B. cereus* biovars anthracis contain plasmids with high similarities to pXO1 and pXO2 plasmids. The strains are associated much with the tropical African region (Antonation *et al.*, 2016). The phylogenetic analysis shows that these strains are different from *B. anthracis* and investigation based on their plasmids suggest that they have gained these plasmid once by *B. cereus* biovars anthracis ancestor which persisted within the lineage therefore, this rule out the possibility of genetic manipulations (Antonation *et al.*, 2016; Baldwin, 2020).

An additional virulence attribute observed among these strains is the presence of an active capsule. The capsule, which is composed of hyaluronic acid, is pXO1-encoded, and is inactive in *B. anthracis* due to frame-shift mutation on pXO1 (Antonation *et al.*, 2016). On microbiological features the strains shares intermediate properties with *B. cereus* (motility, penicillin resistance and gamma phage) and *B. anthracis* (non-hemolytic). (Antonation *et al.*, 2016; Hoffmaster *et al.*, 2004; Hoffmaster *et al.*, 2006; Cloeckaert *et al.*, 2020).

Another study presented *Bacillus cereus* biovars *anthracis* strain CI that harbors the plasmid with virulence genes of *B. anthracis* on its two plasmids namely pCI-XO1and pCIXO2 and they are highly syntenic and indicated 99% to 100% identity to pXO1 and pXO2 of *B. anthracis* plasmids respectively. In the coding regions of these plasmids there were no differences identified. While difference in pCI-XO1 and pXO1 were noted, these differences were not greater than the differences among the plasmids of *B. anthracis* (Klee *et al.*, 2010). Anthrax cases rarely occur in primate except in humans (Leendertz *et al.*, 2006). It is therefore exceptional to find apes in the rainforest region that were killed from anthrax-like infection by *Bacillus cereus* biovars *anthracis* strain CI. This was first described as at least six chimpanzees died in Tai National Park, Ivory Coast died between 2001 and 2002 (Leendertz *et al.*, 2006; Klee *et al.*, 2006; Klee *et al.*, 2010).

These strains were sequenced, targeting the 16S rDNA gene which indicated them to be members of the *B. cereus* group (Liu *et al.*, 2017; Klee *et al.*, 2006) that contained plasmid-borne genes found in *B. anthracis*. Based on this, it was concluded that two different but related *B. anthracis* were responsible for the infection of chimpanzees in Cameroon and Ivory Coast (Antonation *et al.*, 2016). However genetic analysis showed them to be

different from *B. anthracis* strains as the strain formed a different cluster from all described *B. anthracis* strains at that time. Real-time PCR revealed the presence of *pag* gene in all sub clones which confirmed isolates to be *B. anthracis*-like (Klee *et al.*, 2006; Leendertz *et al.*, 2006).

BLAST search revealed *B. cereus* biovars CI and CA strains to have high homology (Antonation *et al.*, 2016). The phylogenetic relationship of *B. cereus* biovars CI and CA strains to classic *B. anthracis* strains as well as others bacillus strains showed these strains to be closely related to classic *B. anthracis*, *B. thuringiensis* serovar konkukian strain 97-27, and *B. cereus* strain E33L (Han *et al.*, 2006). The virulence contributing genes *atxA* (transcriptional regulator gene), *pag, lef, cya*, of pXO1 and pXO2 were also confirmed in these isolates confirmed (Klee *et al.*, 2010; Leendertz *et al.*, 2006).

These strains also have a relationship with *B. cereus* strain E33L isolated from soil around a dead Zebra that was suspected to have died of anthrax in Etosah National Park, Namibia (Klee *et al.*, 2006; Han *et al.*, 2006). *B. cereus* E33L contains two large and three small plasmids (Han *et al.*, 2006; Klee *et al.*, 2010) but they don't encode the virulence factor found in *B. anthracis*, or in anthrax-like isolates, or *B. thuringiensis*. Generally *B. cereus* bv CI and CA strains plasmids are named pBCXO1 and pBCXO2 and their chromosomal background is more close to *B. cereus* and *B. thuringiensis* strains (Cloeckaert *et al.*, 2020; Klee *et al.*, 2010; Antonation *et al.*, 2016; Leendertz *et al.*, 2006).

2.4.2. *B. cereus* JF3964

This strain was isolated from bovine in Cameroon. Although mostly of the bacillus isolates from anthrax-like infection were from primate in Central Africa (Klee *et al.*, 2010; Klee *et al.*, 2006). JF3964 was isolated from bovine and it showed to be more related to *B.*

cereus biovar anthracis strain CI. At the time of its isolation, JF3964 showed a profile that is not similar to those that has been described and it was grouped into a novel clade based on Pilo, et al., 2011 and placed on a new cluster determined by multiple loci VNTR analysis (MLVA). *B. cereus* biovar anthracis strain CI (isolate from a chimpanzee from the Ivory Coast) (Antonation *et al.*, 2016; Klee *et al.*, 2010) was the most closely related to *B. cereus* JF3964 but was different from it (Pilo *et al.*, 2011; Klee *et al.*, 2010). Generally Pilo, et al., (2011) concluded that the strain *B. cereus* JF3964 isolated from Cameroon is grouped among those isolated from the same region (Central Africa) and those isolates (example, *B. cereus* biovar anthracis strain CI and *B. cereus* biovar anthracis strain CA) have not been isolated elsewhere since that time.

2.4.3. *B. cereus* G9241

B. cereus G9241 was associated with pneumonia in a welder in 1994. The isolate was recovered from blood and sputum of a patient with life-threatening pneumonia (Hoffmaster *et al.*, 2006; Hoffmaster *et al.*, 2004). The patient had symptoms of inhalation anthrax and the etiology agent harbors almost pXO1 virulence plasmids and its genome share homology with *B. anthracis*. In addition, *B. cereus* G9241 have pBC218 that encode for a novel polysaccharide capsule operon and it is responsible for the capsule detected on this isolate (Hoffmaster *et al.*, 2004; Baldwin, 2020).

The isolate G9241 contained protective antigen (*pagA*) gene homologous to *B. anthracis* pXO1-encoded (Hoffmaster *et al.*, 2006; Wilson *et al.*, 2011). However, the isolate does not contain pXO2-encoded gene responsible for the biosynthesis of capsule of *B. anthracis*. Analysis also showed the pXO1 gene PA 99.7%, LF (lethal toxin) 99%, and (EF) edema factor 96% identical to those genes of *B. anthracis.* The regulatory protein

known as AtxA is 100% (Hoffmaster *et al.*, 2006) identical to that of *B. anthracis* (Wilson *et al.*, 2011; Scarff *et al.*, 2016; Hoffmaster *et al.*, 2004). The *pagA* gene is different from that of *B. anthracis* due to two point mutation found in its sequences (Marston *et al.*, 2016; Hoffmaster *et al.*, 2006). The isolate did not react to *B. anthracis*-specific capsule antibodies, although it expressed a capsule. The expressed capsule did not require CO_2 as it is normally with *B. anthracis* but in the presence of CO_2, the capsule was rather thicker compared to its absence. A novel gene cluster of polysaccharide capsule biosynthetic gene is observed in pBC218 plasmid of *B. cereus* G9241 (Hoffmaster *et al.*, 2004). This plasmids also encoded for pXO1-encoded toxin regulator AtxA, PA, and LF (Brézillon *et al.*, 2015) with different percentage similarities that are observed on pBCXO1 plasmid as they are both compared to pXO1 of *B. anthracis*. The isolate does not encode for genes for pXO2-encoded poly-D-glutamic acid capsule of *B. anthracis* and it also do not encode for edema factor gene on pBC218. G9241 isolate was tested for its lethality in mice and it was 100% as the positive control *B. anthracis* Sterne (Hoffmaster *et al.*, 2004; Hoffmaster *et al.*, 2006).

2.4.4. *B. cereus* 03BB87, 03BB102, 03BB108

B. cereus 03BB87 was isolated from a muller operator, Texas, United States. While *B. cereus* 03BB102 and 03BB108 were isolated from a welder and from his worksite, respectively (Hoffmaster *et al.*, 2006). Generally the isolates (*B. cereus* 03BB87, 03BB102, and 03BB108) and the Louisiana isolate *B. cereus* G9241 (Hoffmaster *et al.*, 2006; Hoffmaster *et al.*, 2004) were closely related to *B. anthracis* yet they were distinct from to it. The specific primers for *B. anthracis* chromosomal sequences did not amplify on any of these isolates (*B. cereus* 03BB87, 03BB102, 03BB108, or G9241). These

isolates are found in the branch of the phylogenetic tree that has a lot of the known pathogenic and virulent *B. cereus* and *B. thuringiensis* isolates (Klee *et al.*, 2006; Han *et al.*, 2006). However, protective antigen gene (*pagA*) (Marston *et al.*, 2016) was detected in *B. cereus* 03BB87 and 03BB102 by PCR. The identified *pagA* sequence in *B. cereus* 03BB87 was similar to that of *B. cereus* G9241 (Hoffmaster *et al.*, 2004; Hoffmaster *et al.*, 2006). The identified *pagA* gene was not identical to others and are distinguishable from those that were sequenced at that time because of two point mutations that were identified (Hoffmaster *et al.*, 2006).

B. cereus 03BB87 could not be differentiated from *B. cereus* G9241 isolate based on AFLP (Amplified fragment length polymorphism) and MLST (Multilocus sequence typing) analysis (Han *et al.*, 2006; Kubo *et al.*, 2011; Hoffmaster *et al.*, 2006). *B. cereus* 03BB87 also revealed identical PCR profile for pBC218 as in *B. cereus* G9241 which indicated the presence of this plasmid on the isolate (Hoffmaster *et al.*, 2006). *B. cereus* 03BB87 have a putative polysaccharide polymerase gene and this was not detected in *B. cereus* 03BB102 and 03BB108. The only difference expressed between *B. cereus* 03BB87and *B. cereus* G9241 was the degree of capsule expression although they both have the pBC218 operon for capsule formation (Hoffmaster *et al.*, 2006; Hoffmaster *et al.*, 2004). The analysis of *B. cereus* 03BB87 and *B. cereus* G9241 of pXO1 plasmid were identical based on the amplification of open reading frames (ORF) on the plasmid. Both 03BB87 and G9241 have no enough homology to any of pXO2-specific ORF fragments (Hoffmaster *et al.*, 2006; Hoffmaster *et al.*, 2004; Baldwin, 2020).

B. cereus 03BB102 and 03BB108 isolated from a welder and from his worksite, respectively are similar to but distinct from each other. The isolate are phylogenetic more

similar to virulent bacillus isolates *B. thuringiensis* 97-27 and *B. cereus E33L* isolates (Han *et al.,* 2006; Hoffmaster *et al.,* 2006). The isolates also have few homologous sequences *B. anthracis* pXO1 and pXO2 plasmids. *B. cereus* 03BB102 and 03BB108 harbors *capA,* cap*B,* and *capC* genes (Hoffmaster *et al.,* 2004; Scarff *et al.,* 2016; Hoffmaster *et al.,* 2006)and this may play a role in pathogenesis just as in *B. anthracis*, as also one isolate (*B. cereus*) caused fatal infection in health individual, although it does not harbor *pagA* gene Miller et al. as sited in, (Hoffmaster *et al.,* 2006).

The analysis also revealed that *B. cereus* 03BB108 seems to be a subset of *B. cereus* 03BB102 (Hoffmaster *et al.,* 2006). *B. cereus* 03BB102 contained pXO1 ORFs (open reading frames) that were not detected in *B. cereus* 03BB108. *B. cereus* 03BB102 tested positive for gene from pXO1 pathogenicity island (Hoffmaster *et al.,* 2006). These genes includes the tripartite toxin genes (*pagA, lef,* and *cya*) (Baldwin, 2020). This is different from *B. cereus* 03BB108 (environmental isolate) as majority of ORFs of pathogenicity island are missing. However these two isolate revealed the presence of ORF of pXO2 mainly *capA*, *capC*, and *capB* genes. These genes are responsible for biosynthesis of capsule in *B. anthracis* (Hoffmaster *et al.,* 2006; Brézillon *et al.,* 2015; Wright *et al.,* 2011).

2.4.5. *Bacillus* cereus Elc2

This isolate was cultured from a welder with inhalation anthrax-like infections. The genome of *Bacillus cereus* Elc2 share greatest similarity with *B. anthracis* Ames Ancestor, *B cereus* 03BB102 and *B thuringiensis* Al Hakam isolate. The isolate also contained pBCXO1 plasmid (Wright *et al.,* 2011; Hoffmaster *et al.,* 2006).

2.4.6. *B. cereus* isolate, BcFL2013

The BcFL2013 isolate was the first to be isolated from anthrax-like cutaneous lesion. It was isolated from a patient with skin eschar (Marston *et al.*, 2016). The isolate was beta-hemolytic on blood agar (Klee *et al.*, 2006; Marston *et al.*, 2016). Polymerase chain reaction (PCR) analysis indicated that *B. cereus* BcFL2013 is similar to *B. cereus* G9241 isolate (Marston *et al.*, 2016; Hoffmaster *et al.*, 2004; Hoffmaster *et al.*, 2006). This was based on the fact that isolates like *B. cereus* G9241 was positive for pXO1-encoded tripartite genes (*pagA, lef,* and cya) and negative for capsule gene encoded in pXO2 (Hoffmaster *et al.*, 2006). Contrary BcFL2013 was not positive for pBC210 as it is the case with G9241 and 03BB87 (Marston *et al.*, 2006; Hoffmaster *et al.*, 2006). Based on whole genome sequence of the isolate it indicated that the isolate contained the pBCXO1 plasmid with about 2.5 kb deletion (Hoffmaster *et al.*, 2004; Brézillon *et al.*, 2015; Marston *et al.*, 2016). The comparison on pBCXO1 was >99.98% similarity (Marston *et al.*, 2016). In fact, the toxin gene on pBCXO1 plasmid comparison yielded 100% similarity. BcFL2013 shown to have about half of the pBC210 of G9241 (Scarff *et al.*, 2016; Marston *et al.*, 2016). This reads did not include the operon responsible for capsule biosynthesis. But, the 108,352 kb homologous to pBC210 have an average nucleotide identity of 99.84% in comparison G9241 (Marston *et al.*, 2016).

2.4.7. *Bacillus thuringiensis* serovar konkukian strain 97-27 and *Bacillus cereus* E33L

These two strains are among virulent isolates in the *Bacillus* genus. They don't have plasmid-borne virulence genes found in *Bacillus anthracis*. However these strains are found to be more related to anthrax-like isolates (Hoffmaster *et al.*, 2006; Klee *et al.*, 2006; Baldwin, 2020). *Bacillus thuringiensis* serovar konkukian strain 97-27 was isolated from

a human severe tissue necrosis from Paris, France (Hernandez *et al.*, 1998; Han *et al.*, 2006). *Bacillus cereus* E33L was isolated from soil at site of a dead zebra that is suspected to be dead of anthrax in Namibia (Han *et al.*, 2006). Multilocus sequence typing revealed that these two virulent strains of the *B. cereus* group mainly *B. thuringiensis* serovar konkukian strain 97-27 and *B. cereus* E33L are closely related to *B. anthracis* (Han *et al.*, 2006; Klee *et al.*, 2010).

Bacillus thuringiensis serovar konkukian strain 97-27 and *Bacillus cereus E33L* were found to poses virulence genes including five genes that are homologous to virulence genes encoded by the gram positive pathogen *Listeria monocytogenes* (Han *et al.*, 2006). This brings it to the attention that bacillus species may have virulence genes that are still to be discovered or they may have acquired virulence genes from other genus or even among themselves as *Bacillus thuringiensis* serovar konkukian strain 97-27 *and Bacillus cereus* E33L were also found to have cytotoxin K which was from *B. cereus* (ATCC 14579) (Han *et al.*, 2006). *B. thuringiensis* serovar konkukian strain 97-27 harbors *hbl* operon which contain hemolytic enterotoxin genes *hblCDBA* and surprisingly they are found in *B. cereus* (ATCC 14579) and this genes seems to be regulated by the transcriptional regulator *TrrA*, which is part of a large, approximately 17.7-kb, 11-gene insertion. This seemed to be a mechanism for the acquisition of these virulence factors in *B. thuringiensis* 97-27, *B. cereus* 14579, and *B. cereus* G9241 (Han *et al.*, 2006; Hoffmaster *et al.*, 2004). There *plcR* gene is mutated in *B. anthracis* strains but it not in *B. cereus* and it up regulates the transcription of genes such as those involved in motility and chemotaxis, transcriptional regulators and transporters by attaching to a specific

upstream motif. The gene *plcR* is intact in *B. cereus* E33L and in *B. thurigeniss* 97-27 (Han *et al.,* 2006; Antonation *et al.*, 2016).

Although there have been a debate in regard to the systematic classification of *Bacillus cereus* group. These organisms were historically classified into three species which are *B. cereus, B. thuringiensis,* and *B. anthracis,* recent molecular approaches studies revealed similarities between this species (Han *et al.,* 2006). The unifying concept based on sequence analysis is that *B. cereus* group has been derived from the same clonal populations which accounts for the group to be subdivided into consistent phylogenetic clusters. This may suggests the presence of virulence genes shared among these species and also confirming the phylogenetic relatedness of these species (Han *et al.,* 2006; Hoffmaster *et al.,* 2004; Marston *et al.*, 2016)

Among the bacillus species there are also other lineages such as tolworthi, kurstaki, sotto, and thuringiensis lineages but, pathogenic bacillus species such as *B. thuringiensis* 97-27 and *B. cereus* E33L belong to the anthracis lineage and are related to anthrax-like isolates (Han *et al.,* 2006; Hoffmaster *et al.,* 2006). However, even though the relationship of *B. cereus* group is said to be nonlinear and complex that is likely resulting in part from horizontal gene transfer mechanisms there is still a possibility that gene transfers from *Bacillus anthracis* may be limited because the nutrient rich such as mammal host for the vegetative growth would limit their opportunity for genetic transfer. This may as well result in the homogeneity observed in *B. anthracis* species (Han *et al.,* 2006).

2.5. Culturing of anthrax-like isolates and their phenotypic appearance.

Bacillus species are spore forming bacteria and they can be cultured in the laboratory (Ghenghesh *et al.,* 2014). Their colony morphologies can be primarily be used to

differentiate them in culture. To rule out the growth of non-spore forming bacteria, sample can be heated at 65-80 ^{0}C for 10 minutes. The bacillus species would grow at the incubation temperature range of 28 ^{0}C and 37 ^{0}C for 24 hours. Normally the culturing of *B. anthracis* and anthrax-like isolates have been done on blood agar. Blood agar is a differential media and it will give the hemolysis activity of the bacteria (Priyadarshini and Pradhan, 2019; Luis *et al.*, n.d.; Klee *et al.*, 2006). However the present study scope does not includes the phenotypic appearance or colony morphologies of bacillus species but, the phenotypic characteristics of bacteria are always involved in the sub-culturing of bacteria.

The biochemical and physiological characteristics of *B. anthrax* is used for its isolation from clinical samples in clinical laboratory (**Table 2.2**). The colony appearance and antibiotic susceptibility of *B. anthracis* allow the rule out of other bacillus species (Klee *et al.*, 2010; Hoffmaster *et al.*, 2004; Hoffmaster *et al.*, 2006). Example, *B. anthracis* is different from *Bacillus cereus* (the most anthrax-like isolated species) because the latter is normally resistant to beta-lactams such as penicillin (Amesh, 2011). Therefore, colony morphologies and phenotypic characteristics is a good starting point in the search for anthrax-like etiologic agents. The phenotypic, appearance of some anthrax-like isolates will be explained below although the isolation process cannot be limited to these phenotypic characteristics.

The isolated *B. cereus* strains specifically *B. cereus* 03BB87, 03BB102, and 03BB108 (Hoffmaster *et al.*, 2006) presented phenotypic features that differentiated them from *B. anthrancis*. The strains were hemolytic, motile, as expected for *B. cereus*. In addition strains were resistant to gamma phage. The isolates presented different colony

morphologies. *B. cereus* 03BB102 (from a welder) have small, convex, smooth colonies which were hemolytic on blood agar. *B. cereus* 03BB87 (Muller operator) was also hemolytic but the hemolysis zone was smaller than *B. cereus* 03BB102. *B. cereus* 03BB87, the colony morphology is flat, tan, large and granular colonies. *B. cereus* 03BB108 (welders worksite) is intermediate in size, umbonate (raised center), granular and tan (Hoffmaster, et al., 2006). *B. cereus* G9241 was shown to be motile, hemolytic phage resistant, and penicillin resistant (Hoffmaster *et al.*, 2004) all these features contradicts *B. anthracis*.

Bacillus cereus BcFL2013 (**Table 2.2**) is beta-hemolytic on blood agar, gamma phage resistant with colony morphologies that are typical for *B. cereus*. The isolate have umbonate, large, dark tan and granular colonies and these is also the same as *B. cereus* 03BB108 (Marston *et al.*, 2016; Hoffmaster *et al.*, 2004). Specifically *B. cereus* biovar anthracis strain CI showed motility (Klee *et al.*, 2010), it also present phenotypic features that are similar with strain *B. cereus* JF3964 which includes resistant to penicillin and phage (Pilo *et al.*, 2011). Strain *B. cereus* JF3964 present symptoms of anthrax in cattle and it is thought to be similar to *B. cereus* biovar anthracis strain CI (Pilo *et al.*, 2011).

The capsule biosynthesis is not usually produced in ambient atmosphere by *B. anthracis* although this was a case with some anthrax-like isolates. The CI and CA strains (**Table 2.2**) produce a capsule on ambient condition. The capsule was detected in *B. anthracis* strain and the CI and CA strains under atmosphere enriched with CO_2. In addition the capsule was identified under ambient atmosphere in Columbia blood agar. The CI were sensitive to penicillin while CA were resistant (Klee *et al.*, 2006). This phenotypic features

and biochemical reactions show that anthrax-like isolates cannot be limited to certain test in comparison to *B. anthracis* during laboratory diagnosis.

To add, *B. cereus* biovar anthracis (Antonation *et al.*, 2016) and other anthrax-like isolates present microbiological features that are generally uncommon to *B. anthracis* although they tested positive for *B. anthracis* genes. This is based on differential diagnostic criteria that is also used at central veterinary laboratory (CVL) in Windhoek, Namibia. Moreover, differential based *B. anthracis* criteria appear based on the capsule production of anthrax-like isolates, because they produce a layer of capsule on ambient atmosphere (Antonation *et al.*, 2016; Cloeckaert *et al.*, 2020; Klee *et al.*, 2010; Klee *et al.*, 2006; Hoffmaster *et al.*, 2004).

Generally, the anthrax-like isolates present diverse of phenotypic features. There are those that present some phenotypic features as those of *B. anthracis*, while others contradict *B. anthracis* (Baldwin, 2020; Klee *et al.*, 2006; Leendertz *et al.*, 2006). All in all, according to phenotypic appearance it is possible to rule out *B. anthracis* and knowing the phenotypic features of anthrax-like isolates will be a good starting point in searching for anthrax-like isolates (Klee *et al.*, 2006; Hoffmaster *et al.*, 2006; Saikia *et al.*, 2019; Baldwin, 2020). As it is the case with *B. anthracis* and other clinical important bacterial species the phenotypic and biochemical reactions will be of great use in diagnostic laboratory and identification process.

2.6. Identification of bacteria and sequence analysis.

Firstly, the critical point in bacteriology is the proper identification of bacteria (Chauhan *et al.*, 2020). Isolated bacterial strains can be identified due to their unique characteristics based on molecular (Gajdács, 2020) and phenotypic features. Bacterial colonies can have

specific color, size, elevation, smoothness or roughness of surfaces and spreading for specific isolated strains. In addition different bacterial isolates can be differentiated based on biochemical reactions which may give the identification at the genus level (Chauhan *et al.*, 2020; Anon, n.d.). The widely used method of bacteria identification is the combination of molecular, biochemical reaction and phenotypic (colony morphology and cellular morphology). This combinations is also the method used in the *Bergey's Manual of Systematic Bacteriology* which is widely accepted by Microbiologist (Clarridge, 2004).

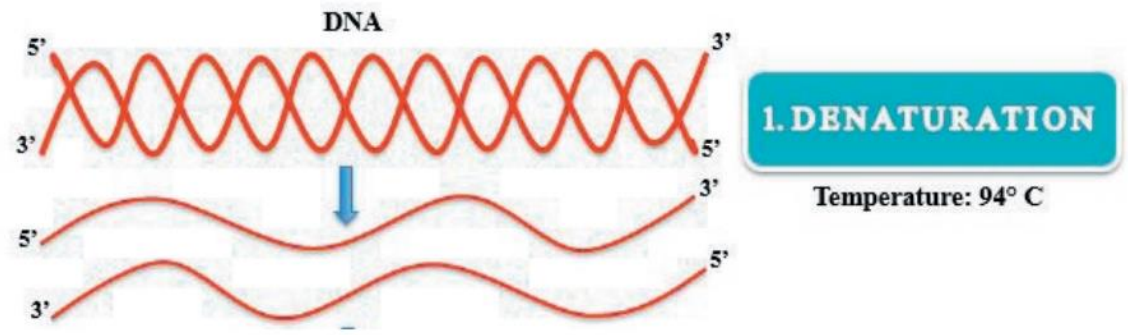
Bacteria can be grouped into two groups based on gram staining reactions (Smith and Hussey, 2005). The gram stain is based on cell-wall composition mainly the peptidoglycan content. The two main groups based on this stain is called Gram-positive and Gram-negative bacteria. This biochemical test is the most used in the identification of unknown bacteria isolate (Chauhan *et al.*, 2020). As for this study, *Bacillus* is a genus of gram positive bacteria as explained in Chapter 2. After gram staining and biochemical tests DNA extraction may be carried out and be used in the identification of the bacteria.

2.6.1. PCR (Polymerase Chain Reaction)

Polymerase chain reaction is a process of copying DNA fragment into multiple copies (Ehtisham *et al.*, 2016). It is an in vitro replication of specific DNA fragment that uses a DNA polymerase enzymes. It was invented in 1983 by Mullis (Kaunitz, 2015; Garibyan and Avashia, 2014). The DNA amplification by PCR is carried out in a number of cycles (Kaunitz, 2015). Each cycle have three steps namely denaturation, primers annealing/hybridization and elongation/extension. Below is an explanation of the steps in each cycles (Ehtisham *et al.*, 2016; Kadri, 2020).

1. Denaturation

DNA strands are hold together by hydrogen bonds, therefore to initiate the replication process DNA strand are separated by heating at the denaturation temperature of 94°C because the hydrogen bond cannot be maintained at the denaturation temperature (Ehtisham *et al.*, 2016; Kadri, 2020; Garibyan and Avashia, 2014).

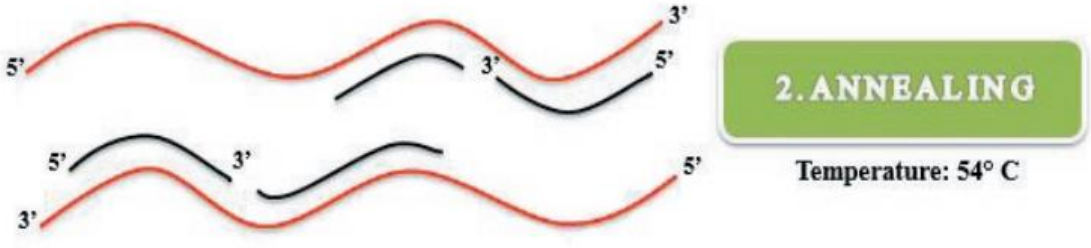


Source: (Ehtisham *et al.*, 2016)

Figure 2.1: Figure illustrate the denaturation process at 94°C.

2. Annealing/Hybridization

This is the second steps after elongations. This process involves the annealing of the oligonucleotide primers to the template DNA or complement DNA. Primmer hybridization is crucial for the thermostable DNA polymerase to copy the complement DNA. The hybridization temperature is normally called primer hybridization temperature. The following figure gives an illustration on this step (Ehtisham *et al.*, 2016; Kadri, 2020; Garibyan and Avashia, 2014).



Source: (Ehtisham *et al*., 2016).

Figure 2.2: Figure illustrate the annealing of oligonucleotide primers in PCR. The annealing temperature (40 to 70°C) is dependent on the primers and can be optimized based on primers. In this illustration the annealing temperature is optimized to 54°C.

3. Elongation/extension

This process is the last step in PCR. The elongation temperature is 72°C, in this step the thermostable DNA polymerase catalyze elongation of new DNA strand using the primed single-stranded DNA as a template DNA (Ash *et al.*, 1991). The Taq polymerase is widely used in PCR because it is stable at high temperatures. The process of elongation is carried out by the addition of dNTPs downstream of the primers by the DNA polymerase (Kadri, 2020; Ehtisham *et al.*, 2016).

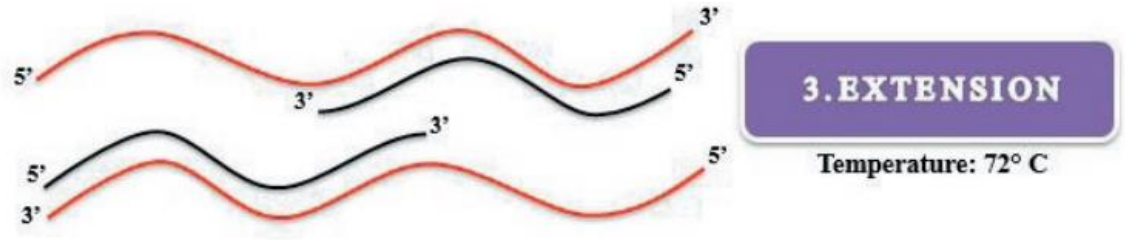


Figure 2.3: Figure illustrates the elongation or extension process during PCR.

After PCR is carried out the amplified DNA can be visualized and analyzed by gel electrophoresis. The amplified DNA can then be used for the desired experiment including for example bacteria identification in cases when 16S rDNA is amplified (Ehtisham *et al.*, 2016; Kadri, 2020; Anon, n.d.; Ash *et al.*, 1991).

2.6.2. 16S rRNA gene/16S rDNA for bacteria identification and taxonomy

The science of classification of living organisms is called taxonomy. Taxonomy is derived from Greek words *taxis and nemein. Taxis* means arrangement or odder while means *nemein*- to distribute or govern. Bacteria taxonomy consist of three independent interrelated disciplines which are classification, nomenclature and identification (Garg, n.d.; Parker *et al.*, 2019). For species identification one can use molecular identification (Ma *et al.*, 2018; Parvathi *et al.*, 2009). Molecular microbiology (Anon, n.d.; Chauhan *et al.*, 2020) and sequencing methods have transformed the bacterial taxonomy (Gajdács, 2020). This method is a faster and precise method and it is broadly used in biology including in anthrax-like infections investigations (Ash *et al.*, 1991; Hoffmaster *et al.*, 2004).

16S rRNA gene can be applied in taxonomy and evolutionary studies (Horner and Pesole, 2004). This gene is said to provide accurate results to the level of bacterial genus and in some cases species level (Rizal *et al.*, 2020). 16S rRNA gene is also referred to 16S rDNA,

it has some variable regions which serve as signature for species of bacteria. 16S rRNA encoding gene is highly conserved among the bacteria because it has an essential role in ribosome assembly. Foremost, 16S rDNA is a good molecular chronometer (Ash *et al.*, 1991), due to its nature and the degree of its conservation is a results of its importance in the cell function as compared to other genes translated into enzymes, since mutation in those genes can be tolerated frequently. Although the rate change of 16 S rDNA is not known to its absolute value it is considered as a good molecular chronometer and one can draw evolutionary relationship distance and relatedness of bacteria from it (Priyadarshini and Pradhan, 2019; Chauhan *et al.*, 2020; Clarridge, 2004). Based on the variable regions, 16S rDNA is an ideal DNA fragment used for identification of bacteria, comparative, phylogenetic studies and classification (Priyadarshini and Pradhan, 2019; Chauhan *et al.*, 2020).

To identify bacteria by molecular methods one have to extract DNA from bacterial cells. Once DNA is extracted, fragment that encode for 16S rRNA can be amplified by PCR (Muthukumar *et al.*, 2008) and be sequenced. There are universal primers used in the amplification of 16S rDNA which include 27F AGAGTTTGATCMTGGCTCAG Forward and 1492R GGTTACCTTGTTACGACTT Reverse primers (Chauhan *et al.*, 2020). A band of nearly 1.5 kbp can be obtained from PCR and this can be analyzed on gel electrophoresis (Chauhan *et al.*, 2020; Clarridge, 2004). However 16S rRNA gene is not without shortcomings. It have been indicated that 16S rRNA have poor discriminatory power for some genera and this includes some bacillus species (Janda and Abbott, 2007).

Nowadays, bacteria identification is done using molecular methods especially 16S DNA sequence together with biochemical tests. Specifically the use of 16S DNA have been

used in applications to identify bacteria from environmental samples, clinical samples (Maleki *et al.*, 2020; Muthukumar *et al.*, 2008; Clarridge, 2004; Ash *et al.*, 1991) and other samples such as food samples (Ceuppens *et al.*, 2014). Of consideration molecular methods have advantages over biochemical methods although it may also have shortcomings. In studying bacillus species isolated from anthrax-like disease molecular methods have been used in the identification of bacterial isolates and screening for virulence genes (Chauhan *et al.*, 2020).

3. CHAPTER THREE: RESEARCH METHODS

3.1. Study design

The study was carried out at the Central Veterinary Laboratory (CVL) in Windhoek. The permit for sample collection, as well as the ethical clearance were obtained from the University of Namibia (UNAM) and Central Veterinary Lab (CVL). The flowchart in **Figure 3.1** below illustrates the design of the whole study.

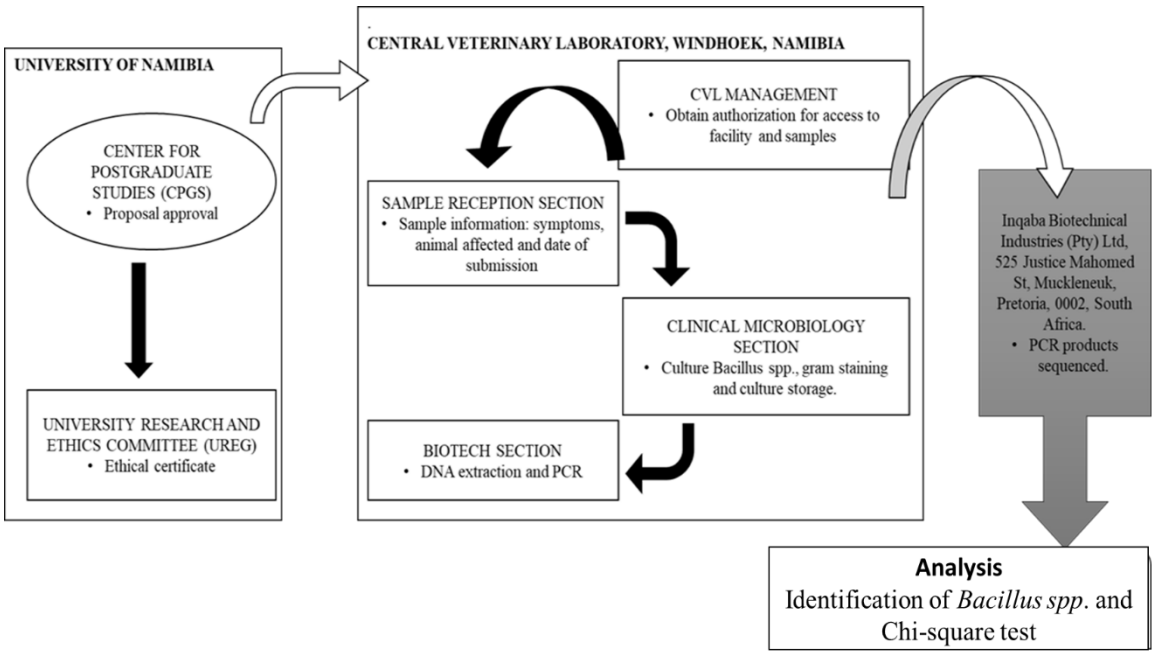


Figure 3.1: Study design. The gray colored box indicates the methods that were carried out in South Africa and therefore the procedures for the gray box are not explained in the research methods.

3.2. Study area

The study was conducted at Central Veterinary Laboratory (CVL) facility located in Windhoek, Namibia. The CVL facility provides analytical and diagnostic services. CVL is a Namibian government institution under the Ministry of Agriculture, Water and Land

Reform. CVL facility operational procedures comply with ISO 17025, and it is audited by external bodies this contribute to the reliabilities of its results. The facility has several subdivisions including the Clinical Microbiology sub-division under Diagnostic Services. One of the duties of Clinical Microbiology is to diagnose anthrax suspected samples. In addition, CVL is a reference laboratory which receives anthrax suspected samples from around the country that may be submitted at Regional Laboratories in Ondangwa, Grootfontein and Gobabis (*Central Veterinary Laboratory FAO South-South Cooperation Gateway Food and Agriculture Organization of the United Nations*, 2020). Therefore, CVL was deemed a good place from which to obtain samples from anthrax-like infections. CVL only receives anthrax suspected samples taken from animals, hence this study only employed animal samples and no human samples are included in this study.

3.3. Collecting of sample and information of anthrax-negative cases.

Sample information was obtained from the sample reception section of the central veterinary laboratory, Windhoek, Namibia. The anthrax negative samples identification numbers were obtained from the Clinical Microbiology section. The sample identification numbers were used to obtain sample information, and the samples considered were those collected between 2019 and March 2020 (**Appendix 1**). Required sample information included the date of sample submission at CVL, symptoms and the name or category of animals where the samples were collected from. The nature of samples included soil sample around dead animals (in cases of blood oozing and soil was sampled), tissue samples and swabs. The study did not include tallowed fat, bone and meat meals which are only tested for anthrax at CVL for animals feed safety.

The labelling of samples was done by denoting each sample's identification number (CVL sample identification number) into a research number using Arabic numerals (**Table 4.1, - 4.4, Appendix 1**). A total of 23 samples were then recruited in this study.

3.4. Isolation of *Bacillus* spp. from anthrax-negative samples by culture methods

3.4.1. Culturing *Bacillus* spp. from anthrax negative samples

All the procedures for culturing of bacteria were carried out in the Clinical Microbiology section (**Figure 3.1**) and biosafety procedures were considered. The culturing procedures and slide smears were prepared in the biosafety II cabinet.

Firstly, samples were heated in distilled water at 65ºC for ten minutes in a water bath to eliminate the non-spore forming bacteria as described by Klee *et al.* (2006). After heating, serial dilutions on each sample were carried out with distilled water at 10^{-1} and 10^{-2} dilution factors. This was followed by inoculation in horse blood agar and incubation at 37ºC for 18-24 hours following the descriptions of (Klee *et al.*, 2006; Luis *et al.*, n.d.). Sub-cultures were then generated from either or both of 10^{-1} and 10^{-2} inoculated petri plates depending on the growth of colonies. The sub-cultures were generated based on different colony features mainly color, hemolysis, colony shape and size, as it was also done in previous studies and due to the fact that anthrax-like isolates present diverse phenotypic features (Klee *et al.*, 2006; Hoffmaster *et al.*, 2006; Saikia *et al.*, 2019; Baldwin, 2020). Once axenic cultures were obtained, they were stored at -20ºC until further use.

3.4.2. Gram staining.

Prior to DNA extraction, isolates were subjected to Gram staining to confirm that they are Gram positive rods, based on general knowledge that *Bacillus* genus is composed of Gram positive rods bacteria (Akula *et al.*, 2005; Priest, 2008). Gram staining was carried out as described by (Smith and Hussey, 2005). Isolates with Gram positive rods were considered in the next procedures while all that were Gram negative subcultures were discarded.

3.5. Bacterial genomic DNA extraction, PCR and gel electrophoresis

3.5.1. Bacterial Genomic DNA extraction

The DNA extraction was carried out at CVL, Windhoek, Namibia in the Biotech Laboratory. DNA was extracted using a Quick-DNA^{TM} Fecal/Soil Mircobe Miniprep Kit following the manufactures protocol. The extracted DNA was subjected to polymerase chain reaction (PCR) analysis, or otherwise stored at -20^{o}C until further use.

3.5.2. Polymerase Chain reaction

The DNA was subjected to PCR amplification targeting the 16 S rDNA gene of bacteria based on its benefit as described in **section 2.6.2**. The primers used were 27F AGAGTTTGATCMTGGCTCAG and 1492R CGTTACCTTGTTACGCTT (Chauhan *et al.*, 2020). Fifty microliter (50 µl) reaction volumes were run for each sample. The premixed BioLabs master mix was used. Extracted DNA was used as template DNA.

Table 3.1: PCR reaction mixtures for the amplification of the 16S rRNA gene

Reagent	Volume per reaction µl
Nuclease free Water	16
2X Master Mix	25
MgCl*	1
1492R	2
27F	2
Template DNA	4
Total	50

*MgCl = Magnesium Chloride

The PCR cycling conditions were as follows: denaturation at 94 ^{o}C for 5 minutes followed by 30 cycles of melting at 94 ^{o}C for 1 minute, annealing at 55 ^{o}C for 1 minute, elongation at 72 ^{o}C for1 minute 30 second. This was then followed by a final extension step at 72 ^{o}C for 5 minutes.

3.5.3. Gel electrophoresis

The PCR amplicons were resolved in 1% agarose gel electrophoresis. The band sizes of successful amplifications were expected to be around 1500 base pairs (Ash *et al.*, 1991) and the gel were taken with a biodoc analyzer biometra (made in Germany) under the UV light while comparing with a 1 kbp molecular marker (Anon, 2019a). The gel pictures were taken with the same instrument (biodoc analyzer biometra).

3.6. DNA Sequencing, sequence editing and identification of *Bacillus* spp.

3.6.1. DNA Sequencing and sequence editing

The PCR products were sent for sequencing at Inqaba Biotechnical Industries (Pty) (Pretoria, South Africa). The resultant chromatograms were manually edited with Chromas Lite 201 for Windows through base calling. The cleaned sequences were saved

and used for blasting to identify the isolates and infer their taxonomy. The following illustrations in **Figure 3.2** and **Figure 4.1** show the sequence editing process.

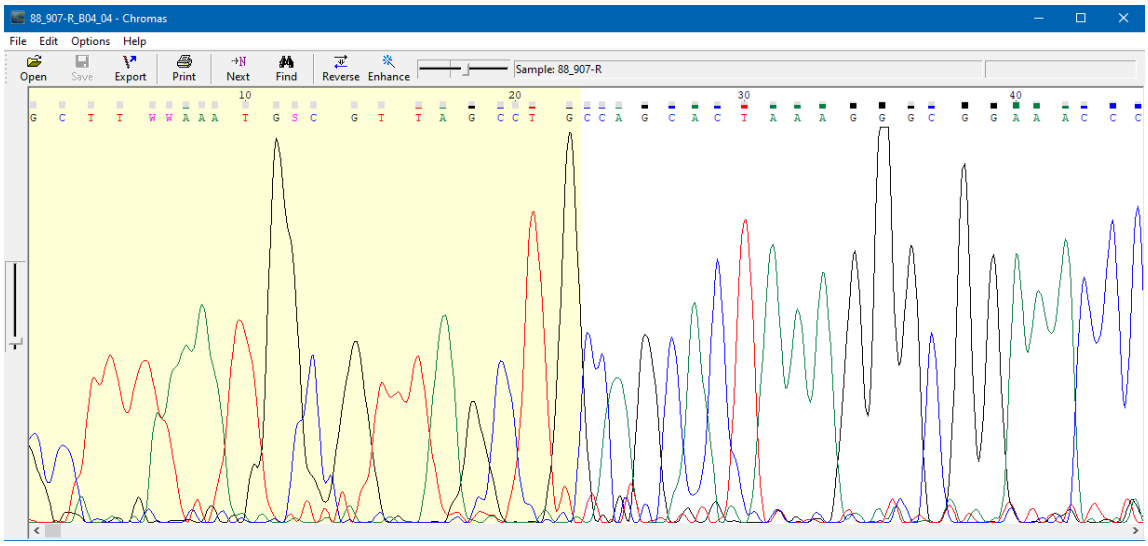


Figure 3.2: Figure shows the Chromas lite window opening a chromatogram for a sequence results, the poor quality reads is highlighted (light yellow on the left hand side) by Chromas.

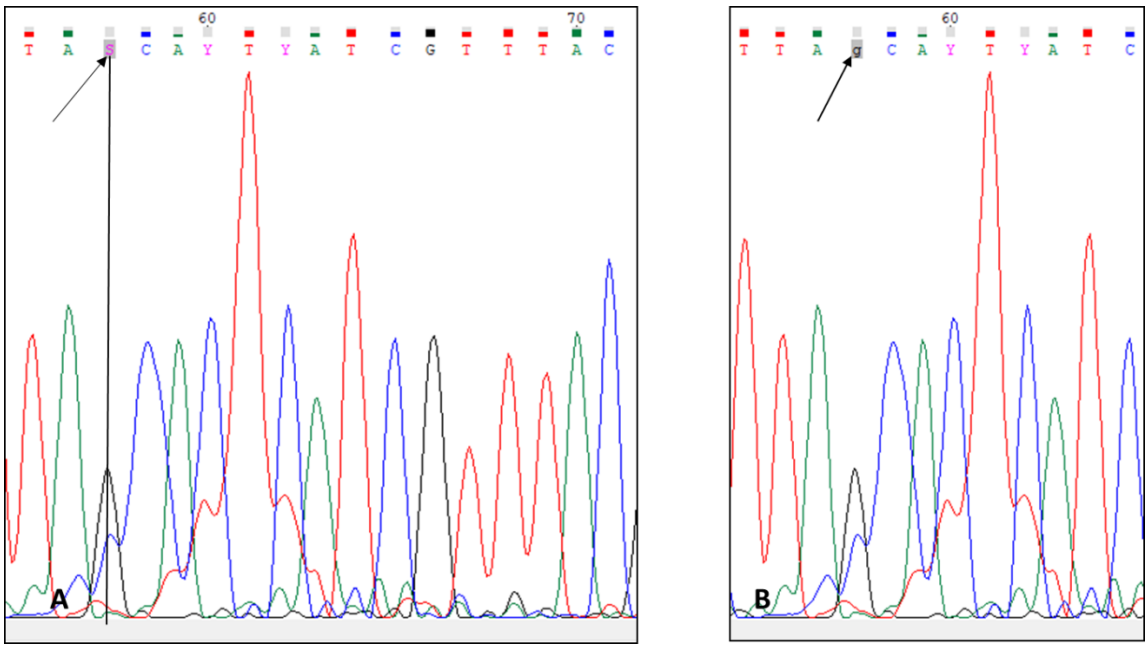


Figure 3.3: Figure showing the chromatogram editing at position 57 pointed by the black arrows in A and B. In A, the sequence redundant (S, redundant appear in pink color throughout the entire sequence) not edited. The vertical line shows that the redundant fit to be G (guanine). In B the redundant is labelled with the correct nucleotide of letter G. The edited positions remain in lower case to know where the manual editions were done in the sequence.

3.6.2. Identification and taxonomy of bacillus species

After manual editing with Chromas lite 201, generated sequences were used for the identification bacillus species on the National Center for Biotechnology Information (NCBI) website using the basic local alignment search tool (BLASTn) tool. The percentage similarity between the query and subject sequences was considered for identification purpose. Percentage identity was also used to confer taxonomy based on the criteria that similarity of 97% represent the same species (Garibyan and Avashia, 2014; Nguyen *et al.*, 2016).

3.7. Data analysis

The test to determine if there was a correlation between reported symptoms and the identified bacteria isolates was carried out on IBM SPSS Statistics 26 using a chi-square test. The chi-square was compared at the significant value of 0.05. The frequencies and percentage value were also calculated in SPSS by descriptive statistics.

3.8. Research ethics

Ethical clearance certificate for this study was obtained from the University of Namibia Research and Ethics Committee (UREC) and Centre for Postgraduate Studies (CPGS). Samples were not collected during the course of the research rather, ready submitted samples were used from central veterinary Laboratory (CVL), and therefore instead of sampling permit, the permission to research on the samples and access to CVL facility was obtained from the CVL management.

4. CHAPTER FOUR: RESULTS

4.1. Samples information.

Samples that were analyzed in the research and their associated symptoms are recorded in **Table 4.1**. In addition, the animals affected were also recorded as per the information provided by CVL. In cases where information such as symptoms and type of animals affected were not provided, and where a broad classification of animals such as "bovine" was given, such samples were still considered for examination. Samples were labeled with research numbers as shown in **Table 4.1**. A total of 12 research numbers are presented here based on sample obtained from CVL and from those research numbers a total of 23 samples were used in the study.

Table 4.1: Sample information obtained from Central Veterinary Laboratory

Research number	Number of samples	DATE RECEIVED	SAMPLE TYPE	Animal affected	SYMPTOMS
1	1	3/3/2020	Blood smear. Tissue	Bovine	No symptoms
2	5	3/9/2019	Tissue	Hippos	Sudden death
3	1	9/9/2019	Tissue	Porcine	Sudden death, Blood oozing from orifices, Rapid peutrification, Bloat
4	2	30/10/2019	Tissue	Caprine	Blood oozing from orifices, Bloat, Sudden death.
5	1	12/9/2019	Tissue	Elephant	Sudden death
6	2	23/08/2019	Tissue	Equine	Acute death
7	2	12/12/2019	Tissue ears	Game (Oryx, Hartebeest	Acute death
8	1	18/05/2019	Brain	Bovine	No symptoms
9	2	15/08/2019	Swab	Kudu Buffalo	Acute death, Blood oozing from orifices

Research number	Number of samples	DATE RECEIVED	SAMPLE TYPE	Animal affected	SYMPTOMS
10	4	8/8/2019	Tissue	Bovine	Sudden death
11	1	8/8/2019	Tissue	Equine	Sudden death
12	1	20/08/2019	Soil	Zebra	Acute death, Blood in the soil around it

Note: In sample 2 there is a record of sudden death of 14 hippos, although only 5 tissue samples were presented for this sample. In sample 7, there is a record of a number of dead game animals which were found spread over a wide area although only two tissue samples were presented to this study. Considering the symptoms presented, the samples with symptoms match to anthrax symptoms (**Table 4.3, Appendix 1**).

4.2. Primary cultures, Sub-cultures and gram staining.

After primary cultures which were done from serial dilutions as explained in the methods, isolates were subjected to Gram staining, and the results are presented in **Table 4.2**. The isolates were labeled by adding a lowercase alphabetical later to the isolate description; this is referred to as isolates label code in **Table 4.2**. For example, there were two Gram positive isolates that were generated from 7:2 therefore the isolates were labelled as 7:2a and 7:2b.

Table 4.2: Sub-cultures that were generated from primary of anthrax suspected cases.

Research Sample number	Isolate description	isolates label code	Gram stain
1	1 dry rough	1a	+
2	2-1 sc mixed for pure	2a	+
5	5 large beta hemolytic	5a	+
7	7 -2 cereus	7:2a	+

Research Sample number	Isolate description	isolates label code	Gram stain
7	7 -2 ground grass	7:2b	+
8	8 sc-1	8a	+
8	8 sc-3	8b	+
12	12 sc-1	12a	+
9	9 sc-1	9a	+
9	9 sc-2	9b	+
9	9 biped	9c	+
9	9 large	9d	+
9	9 white; rough	9e	+
10	10:2 flat white colonies	10:2a	+
10	10:2 small raised colonies	10:2b	+
10	10:2 flat brown	10:2c	+
10	10:3 sc-3	10:3a	+
10	10:4 hemolytic non Raised	10:4a	+
10	10:4 whitish cream	10:4b	+
11	11 rough	11a	+
11	11 flat	11b	+

4.3. PCR results

The gel pictures obtained after resolving PCR products on gel electrophoresis are presented in **Figure 4.1A** and **B**. The amplicons size is illustrated in **Figure 4.1A** for the convenience of space. **Figure 4.1B presents** the gel pictures are cut into small sizes in to display the band of each subculture. The band sizes of the amplified DNA were around 1.5kbp which is also the expected band size of amplifying 16S rDNA (Clarridge, 2004).

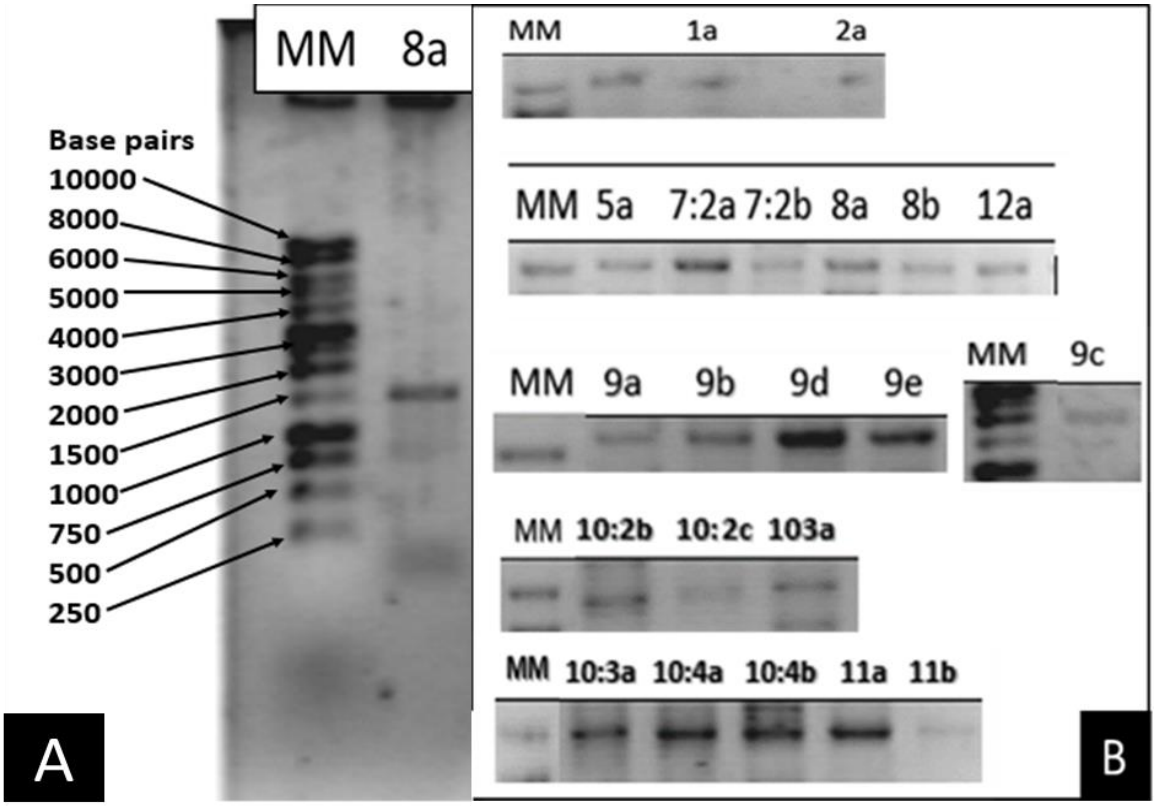


Figure 4.1: A-presents the full picture of gel electrophoresis with a full 1kb molecular maker and the bands of isolate 8a PCR amplicon corresponding to 1.5kbp size based on the labeling shown. B-the gel electrophoresis of PCR product on the generated isolates. The Band size is around 1.5kb based on the 1kb molecular marker as illustrated in **A** of this figure.

4.4. Identification and taxonomy of *Bacillus* spp.

The identified *Bacillus* spp., together with the corresponding symptoms reported in animals from where they were isolated are presented in **Table 4.3**. For additional information see **Appendix 1** that indicated the symptoms of the samples and information on samples on the samples as it was collected from CVL. Based on this study only two symptoms (blood oozing and sudden death see **Table 4.3**) were linked to isolated bacillus

species from samples employed in this study. Therefore the name of the isolated bacteria is as the identification name of the bacillus species in this study.

Table 4.3: The identified bacillus species and the percentage identity of the sequenced DNA of sub-cultures based on the partial sequence of 16S sequences.

Isolate description	**Gel label of sub-cultures**	**Name of isolated bacteria**	**Percentage identity**	**Sequence id**	**Symptoms**
1 dry rough	1a	*Bacillus australimaris*	99.76%	MK256796.1	NSG
2-1 sc mixed for pure	2a	*Bacillus pumilus*	99.88%	MT093483.1	Sudden death
5 large beta hemolytic	5a	*Bacillus subtilis*	99.88%	EU221336.1	Sudden death
7 -2 cereus	7:2a	*Bacillus simplex*	100.00%	MK484265.1	Sudden death
7 -2 ground grass	7:2b	*Bacillus megaterium*	100.00%	MT605458.1	Sudden death
8 sc-1	8a	*Bacillus australimaris*	100.00%	MT510169.1	NSG
8 sc-3	8b	*Bacillus licheniformis*	100.00%	CP025226.1	NSG
12 sc-1	12a	*Bacillus albus*	100.00%	MN960399.1	Sudden death Blood oozing
9 sc-1	9a	*Bacillus megaterium*	99.74%	MT525296.1	Sudden death Blood oozing
9 sc-2	9b	*Bacillus aryabhattai*	100.00%	MT538258.1	Sudden death Blood oozing
9 biped	9c	*Bacillus acanthi*	100.00%	MT588723.1	Sudden death Blood oozing
9 large	9d	*Bacillus thuringiensis*	99.51%	CP050183.1	Sudden death Blood oozing
9 white; rough	9e	*Bacillus cereus*	99.76%	MT611946.1	Sudden death Blood oozing
10 - 2 flat white colonies	10:2a	*Bacillus aryabhattai*	97.74%	MN865859.1	Sudden death

10 - 2 small raised colonies	10:2b	*Bacillus megaterium*	98.59%	MT533921.1	Sudden death
10 - 2 flat brown	10:2c	*Bacillus pumilus*	100.00%	MT093483.1	Sudden death
10-3 sc-3	10:3a	*Bacillus pumilus*	100.00%	MT093483.1	Sudden death
10 -4 hemolytic non Raised	10:4a	*Bacillus zhangzhouensis*	100.00%	MN826587.1	Sudden death
10 -4 whitish cream	10:4b	*Bacillus aryabhattai*	99.88%	MK880629.1	Sudden death
11 rough	11a	*Bacillus subtilis*	99.88%	KY595453.1	Sudden death
11 flat	11b	*Bacillus safensis*	100.00%	KY020051.1	Sudden death

Note: **Apendix 1** contain the same data on symptoms presentment here which are sudden death, and blood oozing. In **Apendix 1** sudden death is a symptoms considered for all deaths and blood oozing is a symptoms deduced from blood in the soil or blood oozing from orifices whereas NSG "no symptoms given" was considered when symptoms are not given. The identified bacillus species are corresponding to their percentage identify to subject sequences. The accession number on NCBI databases of the subject sequences with high percentage is also shown. The symptoms are corresponding to isolated bacillus species.

Each *Bacillus* sp. count on symptoms was also presented on bar graph in **Figure 4.2** the highest count of symptoms per bacillus species isolated was sudden death. Sudden death have three count on three different identified species being *Bacillus aryabhattai*, *Bacillus megaterium,* and *Bacillus pumilus*. *B. cereus* have one count on blood oozing and sudden death.

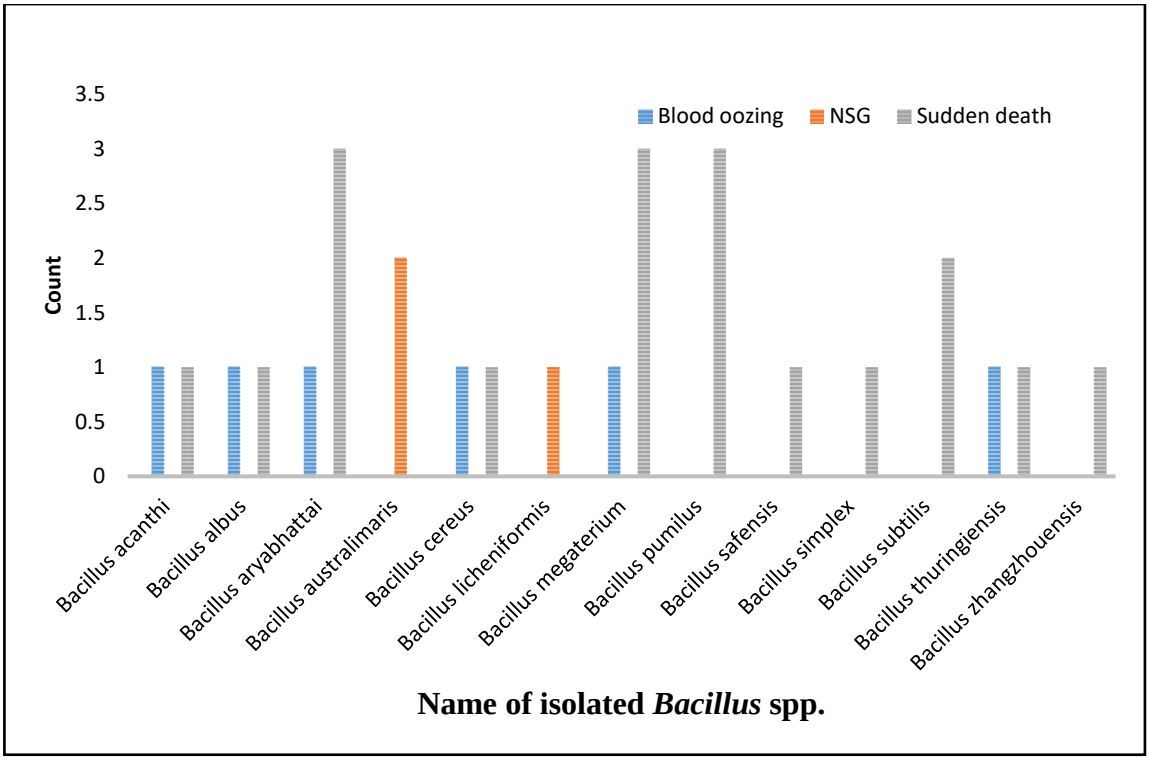


Figure 4.2: The bar graph showing the count number of species isolated appearance on each symptom. The highest count of symptom on identified bacteria was sudden death. NSG: no symptoms given. Note that the bacillus species are those presented in **Table 4.3**.

4.5. Data analysis

Chi-square analysis showed that there was no significant correlation between the isolated bacillus species and presented symptoms (**Appendix 2 and 3**). The chi square value is 0.104 which is greater than the significant value (p=0.05). In addition, the frequencies and percentage value are presented in **Table 4.4**.

Table 4.4: The table presents the frequencies and the percentage per isolated *Bacillus* sp. the highest frequency count and percentage are 3 and 14.3% respectively and the lowest frequency count and percentage values are 1 and 4.8% respectively.

Isolated bacillus species	Frequency	Percent (%)
Bacillus acanthi	1	4.8
Bacillus albus	1	4.8
Bacillus aryabhattai	3	14.3
Bacillus australimaris	2	9.5
Bacillus cereus	1	4.8
Bacillus licheniformis	1	4.8
Bacillus megaterium	3	14.3
Bacillus pumilus	3	14.3
Bacillus safensis	1	4.8
Bacillus simplex	1	4.8
Bacillus subtilis	2	9.5
Bacillus thuringiensis	1	4.8
Bacillus zhangzhouensis	1	4.8
Total	21	100.0

5. CHAPTER FIVE: DISCUSSION

Of the samples that were analyzed, two (sample 1 and 8) of the 23 samples were collected from asymptomatic cases while 21 samples were collected from cases presenting symptoms that are known to be reported in anthrax-like diseases and in anthrax diseases. Specifically, the symptoms recorded in this study were sudden death of animals, blood oozing, and bloating (**Appendix 1 and Table 4.1**). However, only blood oozing and sudden death symptoms were in the analyzed data with *Bacillus* spp. isolated. Therefor these are the only two symptoms analyzed in chi-square because from sample 3 and 4 there were no identification of bacillus species in which bloating was reported (**Appendix 1 and Table 4.1)**. Thus, the sample information provided can suggest that there is a possibility of anthrax-like infections due to the record of symptoms and the deaths of a number of animals in the several instances of same anthrax suspected cases.

Furthermore, of the analyzed samples from which *Bacillus* spp. were isolated sample 4, 9 and 12 all presented both symptoms of blood oozing and sudden deaths, which seemed to be more anthrax-like disease. The sample information presented on this study also showed that the samples came from different animals. The affected animals included both domestic and wild animals which is noted with previously studies on anthrax-like diseases (Pilo *et al.*, 2011; Baldwin, 2020; Klee *et al.*, 2006). From this study, the record of animals that were recorded were, bovine, hippos, porcine, caprine, elephant, equine, oryx, hartebeests, kudu, buffalo and zebra based on the sample information obtained (**Table 4.1** and **Appendix 1**).

A total of 13 different bacillus species were isolated. *Bacillus cereus* only appeared once from the identified subcultures. *B. cereus* strains as explained (**Chapter 2**, **Section 2.4**)

have been isolated from a number of anthrax-like disease from humans and animals (Pilo *et al.*, 2011; Antonation *et al.*, 2016; Hoffmaster *et al.*, 2004; Hoffmaster *et al.*, 2006) and it was the species isolated from Etosha National Park in Namibia from a zebra carcass suspected to have died of anthrax (Han *et al.*, 2006). The *B. cereus* isolated in this study is isolated from a sample with the symptoms of both sudden death and blood oozing although there is no significant dependence observed of symptoms presented with isolated bacteria (**Figure 4.2**), the sample from which this species is isolated from seemed to be more an anthrax-like infection based on these symptoms.

In addition, there were isolation of species that have been isolated from diseases cases previously including anthrax-like infections (Baldwin, 2020; Duncan and Smith, 2011; Tena *et al.*, 2007). *Bacillus pumilus* although was not isolated from animals previously, it was isolated from 3 Spanish shepherded of which 2 are family members (Tena *et al.*, 2007). *B. megaterium* was also isolated from cutaneous anthrax-like infection in human (Duncan and Smith, 2011). This was also puzzling at the time of culturing *B. megaterium* from anthrax-like cutaneous infections because it was thought to be a contaminant (Duncan and Smith, 2011). The previously species that were isolated from anthrax-like infections form up the percentage of 33.3 % of the isolated bacillus species. Specifically these species and their percentage are: *Bacillus cereus* 4.76%; *Bacillus pumilus* 14.3% and *Bacillus megaterium* 14.3% (**Table 4.4**). Of consideration *Bacillus pumilus* and *Bacillus megaterium* are two of the three (the third being *Bacillus aryabhattai*) identified species with the highest percentage observed in this study as it can be seen from **Table 4.4**.

The highest percentage value observed on species isolation in this study was 14.3% (**Table 4.4**). This value was observed on *B. pumilus* and *B. megaterium* which were previously isolated from anthrax-like infections. Literature on both *B. pumilus* and *B. megaterium* did not provide genomic information of these isolates. Therefore, there is no reference if the two species also harbor virulence genes of *B. anthracis* or not. In this study these isolates both have 3 counts on sudden death (the highest count observed) and specifically *B. megaterium* have additional of 1 count on blood oozing (**Figure 4.2**). Even though there is no evidence that this isolate have pXO1-like and/or pXO2-like plasmids or virulence genes responsible for causing anthrax-like symptoms, it seems that these may cause anthrax-like infections but this still need further investigations. Another species that have 14.3% was *Bacillus aryabhattai* (**Table 4.4**) but was not isolated from anthrax-like infections previously. *B. arybhattai* was firstly isolated in 2006 and have been referenced to bioremediation applications (Paz *et al.*, 2016). Moreover *B. arybhattai* is proposed to be used in the production vanillin (Paz *et al.*, 2016). However, the current study present this species to have the highest percentage observed, (**Table 4.4**). In addition, *B. arybhattai* also have the highest of 3 count on sudden death symptom and a count of 1 on blood oozing (**Figure 4.2**) therefore it may also need further investigation when its incident of isolation from anthrax-like suspected increase.

Furthermore, even though there is an indication that *B. pumilus* and *B. megaterium* species isolated in this study can be involved in anthrax-like diseases, this have not been a case in animals but instead it was reported in human (Tena *et al.*, 2007; Duncan and Smith, 2011). *Bacillus* spp. are known to be ubiquitous and they can be found in soil around the globe and in many cases they may be isolated from samples as environmental contaminant.

However, *Bacillus* spp. can be pathogenic to animals and animals as it was seen in several studies (Han *et al.*, 2006; Baldwin, 2020; Klee *et al.*, 2010; Hoffmaster *et al.*, 2006; Wright *et al.*, 2011). However, although isolation of *Bacillus* spp. may be environmental contaminants, further investigations of anthrax negative samples may rule out environmental contaminants while presenting *Bacillus* spp. that may be pathogenic and cause anthrax-like disease in animals and this may include *B. pumilus* and *B. megaterium* species. The symptoms presented in anthrax negative samples may suggest that there may be causative agent of such symptoms and it is more likely to be bacillus species.

Only two *Bacillus cereus* group species were isolated; *B. cereus* and *B. thuringiensis*. *Bacillus cereus* has been isolated in many anthrax-like infections (Antonation *et al.*, 2016; Wright *et al.*, 2011; Hoffmaster *et al.*, 2006; Baldwin, 2020). Yet, it cannot be specified whether *B. cereus* in this study can be an etiologic agent of anthrax-like infection due to less evidence. To rule this out there is need to screen pXO1 and pXO2 virulence genes for any *B. cereus* that would be isolated from anthrax-like infections. *B. thuringiensis* was isolated from infections (Han *et al.*, 2006) but have not been isolated from anthrax-like infections before. Although *B. albus* is proposed to be among the *B. cereus* group (Liu *et al.*, 2017), it has not been isolated or linked to anthrax-like infection before. *B. cereus* group is known to contain pathogenic *Bacillus* spp. and it may be expected to present anthrax-like etiologic agents but anthrax-like etiologic agents may not be limited to this group alone as it is a case with *B. pumilus* and *B. mageterium* (Baldwin, 2020; Saikia *et al.*, 2019; Öncü *et al.*, 2003).

Among the isolated bacteria were also *Bacillus* spp. that have no history of being isolated from anthrax-like isolates. *B. acanthi* was once isolated from soil rhizosphere of a

mangrove plant (Ma *et al.*, 2018) and it has no record of anthrax-like infections or animal infections. In addition, *B. simplex* has been used in plant applications and there is a lot said about it in literature but yet no reference to of this species to be isolated from anthrax-like infections (Kang *et al.*, 2020; Al-Sman *et al.*, n.d.). Furthermore, *B. zhangzhouensis* and *B. australimaris* isolated in this study have reference of being isolated from sea in China (Liu *et al.*, 2016). *B. subtilis* and *B. licheniformis* also were not isolated from anthrax-like infections, rather they suit to be probiotics (Matar *et al.*, 2019; Kafilzadeh *et al.*, 2018). *B. safensis* is one of the closely related to *B. pimulus*, it has not been referenced to anthrax-like infections but rather have other applications (Lateef *et al.*, 2015). Therefore these bacillus species may be merely environmental contaminants. However, further studies may rule out the possible anthrax-like etiological agents which may include the bacillus species that seems to be merely environmental contaminants in this study.

The statistical analysis on the isolated *Bacillus* spp. and the symptoms presented with chi-square at the significant level of 95% in this study lead to the conclusion that identified species have no dependence to symptoms presented (**Appendix 2 and 3**). The chi-square statistic is 0.104 and there was no dependence between isolated *Bacillus* spp. and symptoms presented, thus we reject the assumption that there is a significant dependence between isolated *Bacillus* spp. and symptoms presented. This tells that the symptoms presented are not likely to be as a result of *Bacillus* spp. isolated. While this may be true there may be a possibility that some (amongst the sub-cultures in this study) species are really environmental contaminants while few among these isolates may not be contaminants but rather anthrax-like etiological agents. Therefore, there is more cautions in drawing conclusion, hence there is a need for further investigation but as for the current

study the chi-square analysis did not show dependency between the bacillus species and symptoms.

The identified bacillus have the satisfying percentage similarity (**Table 4.3**). The 16S rRNA gene criteria was used to identification and taxonomy of the bacillus species. The criteria is based on that if the sequence similarity is greater than 97% (as it is also the case in operational taxonomy unit (OTU) (Garibyan and Avashia, 2014) similarities then its similar species (Sharma *et al.*, 2015; Staley, 2006). Most of the identified species have the percentage of 100% with only one with 97% identity (**Table 4.3**). The 16S rRNA gene has been used in the taxonomy of bacteria and has also been referred to as a good molecular clock (Beye *et al.*, 2018; Ash *et al.*, 1991; Clarridge, 2004) in comparing bacterial evolutions. It also provide accurate identification results of bacteria at genus level and in some instances species level (Rizal *et al.*, 2020). However there are also some resolution problem and this includes some bacillus species (Janda and Abbott, 2007; Rizal *et al.*, 2020) but for this study we regard using it and identify bacillus species.

6. CHAPTER SIX: CONCLUSION

The present study has presented here the isolation of *Bacillus* spp. from anthrax suspected samples. Among the isolated *Bacillus* spp. is the possible anthrax-like isolates that may cause anthrax-like infections as it have been seen in other part of the world. The identified bacillus species in this study were *Bacillus australimaris, Bacillus pumilus, Bacillus subtilis, Bacillus simplex, Bacillus megaterium, Bacillus licheniformis, Bacillus albus, Bacillus aryabhattai, Bacillus acanthi, Bacillus thuringiensis, Bacillus cereus, Bacillus zhangzhouensis* and *Bacillus safensis*. Among the isolated bacillus species there is no identical bacillus species isolated elsewhere from anthrax-like infections although this is usually the expectation in investigating anthrax-like infections. Therefore the hypothesis of this study was not retained.

Taxonomy and identification was done in this via sequencing of amplified 16S rDNA gene which were identified by BLASTn searching NCBI and considering percentage identity, thirteen different *Bacillus* spp. were identified. Among the identified species, *Bacillus mageneterium, Bacillus pimilus* and *Bacillus cereus,* were the species that were encountered in anthrax-like infections before. However, the identified *Bacillus* spp. were not associated with symptoms at significant value ($p<0.05$). This indicates that the isolated *Bacillus* spp. may not have caused the symptoms in sampled animals, hence not anthrax-like etiology agents. The most isolated species in this study based on the percentage are *Bacillus mageneterium, Bacillus pimilus* and *Bacillus aryabhattai*.

7. CHAPETER SEVEN: RECOMMENDATIONS

Further investigation on anthrax-like infections should be carried out in Namibia. The current study isolated bacillus species but there was no screening of virulence genes which was the challenge of the current world pandemic of COVID 19, therefore studies should be designed that aim at isolating bacillus species and screening for virulence genes. Furthermore studies that aim to identify and screen for anthrax virulence genes may present the etiologic agent that are highly recommendable for further studies and consideration in anthrax-like disease.

Site sampling and collecting of sufficient information on symptoms may provide all the symptoms involved at sampling sites of suspected anthrax negative cases. It is also recommended that, the testing laboratory should not discard the isolated *Bacillus* spp. (especially *B. cereus* and species that have been encountered in anthrax-like infections) from anthrax suspected sample, rather further investigate on the possibility of causing anthrax-like infections should be carried out. Lastly, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus pimilus* and *Bacillus aryabhattai* may be considered for virulence gene screening.

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APPENDICES

Appendix 1: Sample information obtained from Central Veterinary Laboratory. This Appendix contains information as it appeared from in CVL facility.

Research number	Number of samples	Date received	Sample type	Animals affected	Symptoms
1	1	3/3/2020	Blood smear. Tissue	Bovine	No symptoms.
2	5	3/9/2019	Tissue	Hippos	Sudden death of 14 hippos,
3	1	9/9/2019	Tissue	Porcine	Sudden death, blood oozing from the nose, and mouth, absence of Rigor mortis, rapid peutrification, bloat.
4	2	30/10/2019	Tissue	Caprine	Blood oozing from orifices, bloat, and sudden death.
5		12/9/2019	Tissue	Elephant	Sudden death.
6	2	23/08/2019	Tissue	Equine	Acute death.
7	2	12/12/2019	Tissue ears	Game (Oryx, Hartebeests)	Found dead, spread over wide area, appears like very acute death.
8	1	18/05/2019	Brain	Bovine	No sign.
9	1	15/08/2019	Swab	Kudu Buffalo	Found dead. Blood oozing from nose, anus, and mouth.
10	4	8/8/2019	Tissue	Bovine	Sudden death.
11	1	8/8/2019	Tissue	Equine	Sudden death.
12	1	20/08/2019	Soil	Zebra	Acute death, blood in the soil around it.

Appendix 2: The Chi-Square outputs. The test was compared at significant interval of 95% which show that there is no association between the isolated bacteria and the symptoms. A) Shows the Chi-Square test, B) shows the Symmetric measures outputs. The chi-square value is 0.104 (bold in **Appendix 2A** and **B**).

Chi-Square Tests			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	33.000[a]	24	**.104**
Likelihood Ratio	25.741	24	.366
N of Valid Cases	27		
a. 39 cells (100.0%) have expected count less than 5. The minimum expected count is .11.			

A

Symmetric Measures			
		Value	Approximate Significance
Nominal by Nominal	Phi	1.106	**.104**
	Cramer's V	.782	**.104**
N of Valid Cases		27	

B

Appendix 3: Output of cosstabulation on Chi-Square analysis from SPSS.

Name of isolated *Bacillus* spp * Symptoms presented Crosstabulation						
			Symptoms presented			Total
			Blood oozing	NSG	Sudden death	
Name of isolated Bacillus spp	*Bacillus acanthi*	Count	1	0	1	2
		Expected Count	.4	.2	1.3	2.0
	Bacillus albus	Count	1	0	1	2
		Expected Count	.4	.2	1.3	2.0
	Bacillus aryabhattai	Count	1	0	3	4
		Expected Count	.9	.4	2.7	4.0
	Bacillus australimaris	Count	0	2	0	2
		Expected Count	.4	.2	1.3	2.0
	Bacillus cereus	Count	1	0	1	2
		Expected Count	.4	.2	1.3	2.0
	Bacillus licheniformis	Count	0	1	0	1
		Expected Count	.2	.1	.7	1.0
	Bacillus megaterium	Count	1	0	3	4
		Expected Count	.9	.4	2.7	4.0
	Bacillus pumilus	Count	0	0	3	3
		Expected Count	.7	.3	2.0	3.0
	Bacillus safensis	Count	0	0	1	1
		Expected Count	.2	.1	.7	1.0
	Bacillus simplex	Count	0	0	1	1
		Expected Count	.2	.1	.7	1.0
	Bacillus subtilis	Count	0	0	2	2
		Expected Count	.4	.2	1.3	2.0
	Bacillus thuringiensis	Count	1	0	1	2
		Expected Count	.4	.2	1.3	2.0

	Bacillus zhangzhouensis	Count	0	0	1	1
		Expected Count	.2	.1	.7	1.0
Total		Count	6	3	18	27
		Expected Count	6.0	3.0	18.0	27.0