INVESTIGATION OF ANTHRAX OUTBREAKS IN ANIMALS IN ETHIOPIA: ISOLATION AND MOLECULAR DETECTION OF *BACILLUS ANTHRACIS.*

BY

ABEBE OLANI BULTO

ADVISOR: DR. FUFA DAWO (DVM, MVSc, PhD)

CO-SUPERVISOR: DR. MATIOS LAKEW (DVM, MVSc)

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ADVISOR: DR. FUFA DAWO (PhD)

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ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OFVETERINARY MICROBIOLOGY, IMMUNOLOGY& VETERINARY PUBLIC HEALTH

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Dr. Fufa Abuna	_____________	______________
Chairman (DVM, MSc, Asso.Prof)	Signature	Date
Prof. Gobana Ameni	_____________	______________
External Examiner (DVM, MSc, PhD, Prof)	Signature	Date
Dr. Gezaheagne Mamo	_____________	______________
Internal Examiner (DVM, MSc, PhD, Asso.Prof)	Signature	Date
Dr. FufaDawo	_____________	______________
Major Advisor	Signature	Date
Dr. MatiosLakew	_____________	______________
Co- Advisor	Signature	Date
Dr. GezahegneMamo	_____________	______________
Department chairperson	Signature	Date

APPROVAL

ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OFVETERINARY MICROBIOLOGY,
IMMUNOLOGY& VETERINARY PUBLIC HEALTH

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: **Abebe Olani Bulto** entitled **INVESTIGATION OFANTHRAX OUTBREAKS IN ANIMALS IN ETHIOPIA: ISOLATION AND MOLECULAR DETECTION OF *BACILLUS ANTHRACIS***and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Veterinary Microbiology.

Dr. Fufa Abuna ______________ ______________

Chairman (DVM, MSc, Asso.Prof) Signature Date

Prof. Gobana Ameni ______________ ______________

External Examiner (DVM, MSc, PhD, Prof) Signature Date

Dr. Gezaheagne Mamo ______________ ______________

Internal Examiner (DVM, MSc, PhD, Asso.Prof) Signature Date

Dr. FufaDawo ______________ ______________

Major Advisor Signature Date

Dr. MatiosLakew ______________ ______________

Co- Advisor Signature Date

Dr. GezahegneMamo ____________ ______________

Department chairperson Signature Date

STATEMENT OF THE AUTHOR

First, I affirm that this thesis is my unalloyed work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for (MVSc) degree in Veterinary microbiology at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

Name: ABEBE OLANI BULTO Signature: ______________

College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: _________________________

TABLE OF CONTENTS

CONTENTES **PAGE**

STATEMENT OF THE AUTHOR iii
TABLE OF CONTENTS iv
ACKNOWLEDGEMENTS vii
ABBREVIATIONS viii
LIST OF TABLES x
LIST OF FIGURES xi
LIST OF ANNEXES xii
ABSTRACT xiii
1. INTRODUCTION 1
2. LITERATURE REVIEW 4
2.1. Etiology 4
2.2. Epidemiology 5
2.2.1. Occurrences 5
2.2.2. Public Health Importance 6
2.2.3. Economic significance 7
2.3. Transmissions 7
2.3.1. Transmission in animals 7
2.3.2. Transmission in Humans 8
2.4. Clinical Progressions 9
2.4.1. Clinical Progression in animals 9
2.4.2. Clinical Progression in Humans 10
2.5. Pathogenesis 11
2.6. Spore and vegetative cell survival 13
2.7. Diagnosis 13
2.7.1. Biosafety Procedures 14

2.7.2. Laboratory diagnosis 14
2.7.3. Criteria for laboratory diagnosis of anthrax 15

TABLE OF CONTENTS (*Continued*)

2.7.4. Culture and Gram Stain 16
2.7.5. Biochemical Test 17
2.7.6. Serological Tests 20
2.7.7. MALDI-TOF Mass Spectrometry 21
2.7.8. Molecular diagnosis 21
2.7.9. *Differential diagnosis* 25
2.8. Anthrax Situation in Ethiopia 25
2.8.1. *Reported outbreaks of Anthrax in Ethiopia* 25
2.8.2. *Laboratory diagnostic capacity of anthrax in Ethiopia* 27
2.9. Treatment and Preventions 28
2.9.1. Availability of Anthrax vaccine in Ethiopia 29
3. MATERIALS AND METHODS 31
3.1. Description of the Study Area 31
3.2. Study subjects 32
3.3. Study Design 33
3.3.1. Data Collection Method 33
3.4. Biosafety Procedure 33
3.5. Sample Collection and Transportation 34
3.6. Bacterial isolation and Identifications 35
3.6.1. Gram-stain 35
3.6.2. Cultural Examination 35
3.6.3. Inducing Capsule Production and Giemsa stain 36
3.6.4. *Modified Ziehl-Neelsen stain for spores* 36
3.6.5. *Malachite stain for spores* 37
3.6.6. *Biochemical Tests* 37
3.6.7. Susceptibility to penicillin G 37
3.7. Molecular Detections 38

3.1.1. DNA preparation from bacterial isolates 38
3.7.1. Procedure for DNA extraction from swab and tissue 38

TABLE OF CONTENTS (*Continued*)

3.8. Data Analysis 40
4. RESULTS 41
4.1. Series of case and epidemiological scenario 41
4.2. Cultural examination 42
4.3. Gram-staining 44
4.5. Penicillin susceptibility 47
4.6. Modified Ziehl-Neelsen Stain for Spores 47
4.7. Malachite Green Stain for Spores 48
4.8. Capsule Production and Capsule Staining 49
4.9. Real-Time PCR 50
5. DISCUSSION 52
6. CONCULUSION AND RECOMMENDATIONS 56
7. REFERENCES 58
8. ANNEXES 66

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ABBREVIATIONS

ASM:	American Society for Microbiology
ATCC:	ATCC American Type Culture Collection
BSL:	Biosafety Level
cAMP:	Cyclic Adenosine Mono Phosphate
CDC:	Centers for Disease Control and Prevention
CLSI:	Clinical and Laboratory Standards Institute
CT:	Cycle threshold
DNA:	Deoxyribose Nucleic Acid
EF:	Edema Factor
EDTA:	Ethylene Diamine Tetra Acetic Acid
ELISA:	Enzyme-Linked Immunosorbent Assay
EPHI:	Ethiopian Public Health Institute
FMD:	Foot and Mouth Disease
GDP:	Grand Domestic Product
HIB:	Heart Infusion Broth
IEC:	International Electro Technical Commission
IPC:	Internal Positive Control
ISO:	International standard Operating Procedure
IU:	International Unit
LF:	Lethal Factor
MALDI-TOF_MS	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
MLST:	Multilocus Sequence Typing
MOLF:	Ministry of Livestock and Fisheries
NAHDIC:	National Animal Health Diagnostic and Investigation Center
NVI:	National Veterinary Institute

NTC:	No Template Control
OHP:	One Health Platform
OIE:	World Organization for Animal Health

Abbreviations (*Continued*)

PA:	Protective Antigen
PBS:	Phosphate Buffered Saline
PLET:	Polymyxin Lysozyme EDTA Thallous Acetate
PPE:	Personal Protective Equipment
PPR:	Peste des Petits Ruminants
QMS:	Quality Management system
ORF:	Open Reading Frame
SANAS	South African National Accreditation System
SBA:	Sheep Blood Agar
SETS:	Swab Extraction Tube System
SDS:	Sodium Dodecylsulfate
SNNPR:	Southern Nations, Nationalities, and Peoples' Region
UK:	United Kingdom
VFF:	Victorian Farmers Federation
WGS:	Whole Genome Sequencing
WHO:	World Health Organization

LIST OF TABLES **PAGE**

Table 1: Appropriate specimen and collection methods for animals suspected of anthrax. 15

Table 2: Differential characteristics of *B. anthracis* and *B. cereus*. 17

Table 3: Suitable primers for confirming the presence of the pXO1 and pXO2 plasmids22

Table 4: Animal and human anthrax cases and deaths reported in different parts of Ethiopia. 27

Table 5: Number of samples collected and cultured positive samples from different anthrax suspected outbreak investigations in animals..................................... 43

LIST OF FIGURES **PAGE**

Figure 1: Gram stain result of *B. anthracis* 5

Figure 2: Worldwide anthrax outbreaks reported in livestock, wildlife and humans,. 6

Figure 3: Transmission cycle of anthrax infection in animals and humans. 9

Figure 4: A cow and a sheep that have died from anthrax 10

Figure 5: Colony morphology of *B. anthracis* grown overnight on blood agar.. 16

Figure 6: Capsule Stain. 19

Figure 7: Gamma-phage assay with positive results 19

Figure 8: MALDI-TOF mass spectrometer.. 21

Figure 9: Map of Ethiopia showing suspected anthrax outbreak investigation areas 32

Figure 10: Colony morphology of *B. anthracis* on Sheep Blood agar.. 44

Figure 11: Gram-staining results of *B. anthracis* isolates 45

Figure 12: Motility test:. 46

Figure 13: Susceptibility test. 47

Figure 14: Modified Ziehl-Neelsen stain.. 48

Figure 15: Malachite green stain:. 49

Figure 16: Mucoid colonies of *B. anthracis* on Nutrient Agar and Giemsa stain. 50

Figure 17: Real-time PCR Result. 51

LIST OF ANNEXES **PAGE**

Annex I: Clinical Description 66

Annex II: Criteria for Laboratory Diagnosis of Anthrax 66

Annex III: Procedure for demonstration of *B. anthracis* in stained smears using Gram's stain 66

Annex IV: Procedure for DNA extraction from swabs and tissues samples protocol...... 67

Annex V: Work Flow chart for Real-Time PCR procedure 71

Annex VI: Relative fluorescence vs cycle number and cycle threshold (CT) value for Real-Time PCR results 72

Annex VII: Preparation of nutrient agar containing 0.7% sodium bicarbonate 72

Annex VIII: Giemsa stain preparations 73

Annex IX: Phosphate buffered saline solution 73

Annex X: Susceptibility to penicillin G in Müller-Hinton agar 74

Annex XI: Disinfection, decontamination and discarding contaminated autoclavable and disposable items. 74

Annex XII: photo of dried meat and ear clip collected samples 75

Annex XIII: Photos of different laboratory activities in Biosafety Level- III 76

Annex XIV: Anthrax suspected deaths in hippopotamus but negative for *B. anthracis* in both culture and real-time PCR 77

Annex XVI: Anthrax suspected deaths in Afar Region in sheep and goat but negative for *B. anthracis* in both culture and real-time PCR 78

Annex XVII: Disease outbreak investigation form 79

ABSTRACT

Anthrax is a zoonotic disease caused by *Bacillus anthracis (B. anthracis)*, a gram-positive, non-motile, and spore-forming bacterium. Animal anthrax is an important and zoonotic disease in Ethiopia and only few of those cases were isolated by laboratory. Outbreak investigation of anthrax suspected cases was conducted from November 2018 to June 2019 in south Gonder, Illubabor, Wolayita, Gurage Zone and Zone 4 of Afar region of Ethiopia, where five different outbreaks in different animal species were reported. Purposive sampling method was used in suspected outbreak cases in domestic animals (cattle and goat) and wild animals such as hippopotamus. Anthrax suspected samples were tested under appropriate biosafety and containment. A total of 63 different samples like nasal swab, rectal swab, tissue, and swab from old carcasses and environmental samples were collected, labeled, packed and transported to NAHDIC, Sebeta for further processing. Isolations of *B. anthracis* was conducted by culturing on blood agar, colony characterization, Gram staining, spore and capsule staining and biochemical tests. The molecular detection was conducted by real-time polymerase chain reaction. 9.8% (6/61) of *B. anthracis* was identified from dried meat (Quwanta) samples from outbreak cases in South Gondor, Farta District and the *Bacillus* isolates were non-hemolytic, non-motile and susceptible to penicillin, which is typical of *B. anthracis*. The identified colonies were further confirmed by real-time PCR, where *B. anthracis* virulent plasmid markers namely protective antigen (on plasmid pXO1) and capsule (on plasmid pXO_2) were detected. This is the first report of PCR confirmed anthrax outbreak case from animals in Ethiopia. Anthrax still remains endemic in the country and causes diseases in livestock. A multi-sectoral collaborative approach and capacity buildings are essential for successful prevention and control of the disease and joint outbreak investigation, proper and timely animal immunization.

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Keywords: *Anthrax*, animals, *Bacillus anthracis*, Ethiopia, *outbreak investigation*, Real-time PCR.

1. INTRODUCTION

Anthrax is an acute, febrile disease of warm-blooded animals, including humans. The disease is caused by *Bacillus anthracis (B. anthracis)*, a Gram-positive, non-motile, endospore-forming bacterium (Mock & Fouet, 2001; Shivachandra *et al*., 2016). The name of the bacterium was derived from "anthrakis", the Greek word for coal, because anthrax in humans causes black, coal-like lesions on the skin at the site of inoculation (Inglesby *et al.,* 1999). Anthrax outbreaks in animals in nearly 200 countries are recorded by The World Anthrax Data Site (http://www.vetmed.lsu.edu/whocc/mp_world.htm) a World Health Organization Collaborating Center for Remote Sensing and Geographic Information Systems for Public Health. The types of data recorded by the World Anthrax Data Site are: country-of-origin, anthrax status, vaccination program, species affected, year of outbreak, number of outbreaks during the year, number of cases, number vaccinated and total livestock population (WHOCC, 2005).

Anthrax is a globally distributed disease, having reported from all continents that are populated heavily with animals and humans. The anthrax status of a given country may be classified into one of six categories: hyper endemic/epidemic, endemic, sporadic, probably free, free and unknown. The countries with hyper endemic/epidemic status are more frequent in Africa including Ethiopia, although the status of Egypt is considered as "Probably free". Examples of regions with unknown anthrax status are the polar extremes, the Arctic and the Antarctic (WHO, 2013).

The disease is an important zoonosis posing a public health threat in many regions of the world, particularly in Africa where it is currently recognized as a neglected zoonosis (WHO, 2009). *B. anthracis* is one of the most molecular monomorphic bacteria known and all the known strains have been separated into five categories (allowing for geographical identification) on the basis of variable numbers of tandem repeats in the variable region of the *VrrA* gene (Jackson *et al*., 1979). When nutrients are exhausted, resistant spores form that can survive in soil for decades (Shivachandra *et al*., 2016).

These spores then germinate when exposed to a nutrient rich environment, such as the tissues or blood of an animal or human host.

In Ethiopia based on Livestock Master Plan animal diseases were prioritized to inform decision-makers on the most appropriate allocation of resources to combat the priority diseases; the criteria's were mainly on the impact of the disease on; households and livelihoods, markets and value chains, and intensification pathways in the animal production system(Shapiro *et al*., 2015). Moreover, Epidemiology Directorate of Ministry of Agriculture (MOA) has also prioritized important livestock diseases; accordingly anthrax was prioritized as the third important livestock disease next to PPR and Foot and Mouth Disease (FMD) (MOLF, 2016 unpublished data).

Moreover, the tripartite ministries, Ministry of Agriculture, Ministry of Health and Ministry of Culture and Tourism (through the Ethiopian Wildlife Conservation Authority), in collaboration with development partners established a national One Health Platform (OHP) with various technical working groups. Since the national OHP was established, various activities have been undertaken. One of the tasks was developing a list of priority zoonotic diseases as an entry for a multi-sectoral joint action to reduce and combat the impact of zoonotic diseases on public and animal health as well as on the national economy. The national zoonotic disease prioritization was conducted using a tool developed by CDC and resulted in 5 priority zoonotic diseases for inter sectoral collaboration of which anthrax was the second top priority next to rabies (Pieracci *et al*., 2016).

Animal anthrax is an endemic disease in Ethiopia which occurs in May and June every year (anthrax season) in several farming localities of the country. Although suspected cases of livestock anthrax are reported from several districts, only few of those cases were isolated by laboratory (Shiferaw, 2004) and molecular detection of *B. anthracis* virulent plasmid markers genes were not carried out in the country. To date, both in public and animal health sectors, anthrax outbreak reports are based on history and clinical signs and there is limited information on laboratory confirmed cases at either the

regional or national levels. The general objective of this study was to investigate the outbreaks of anthrax in Ethiopia in 2019.

The main objectives of this study were:

- ✓ To isolate and characterize etiological agent of anthrax, *B. anthracis,* from outbreaks in different animals in Ethiopia.

2. LITERATURE REVIEW

2.1. Etiology

B. anthracis is the causative agent of anthrax. It is a Gram-positive, endospore forming, rod-shaped, non-motile bacterium (Figure 1) that grows on nutrient media, typically sheep or horse blood, under aerobic or anaerobic conditions with an optimal temperature of 35-37ºC (Mock & Fouet, 2001; Shivachandra *et al*., 2016). Colonies appear as irregular, raised, opaque white to grey when grown on blood agar. The colonies are about 2mm in diameter and tacky on teasing with a loop (Spencer, 2003). Colonies cultured on media containing bicarbonate and incubated in elevated carbon dioxide (5-20%) will produce capsule, causing a mucoid phenotype (Spencer, 2003). Under a microscope, the vegetative form of the bacteria appears as square-ended in chains of two or more with an elliptical spore in the middle of the cell (Mock & Fouet, 2001).

Vegetative *B. anthracis* are poor survivors outside of a host and therefore produce endospores to survive long term in the environment (Mock & Fouet, 2001; Moayeri *et al*., 2015). This characterizes *B. anthracis* as an obligate pathogen (Baillie &Read, 2001; Spencer, 2003; WHO, 2008). An endospore is the dormant form of a vegetative cell and formed in the presence of oxygen, towards the ends of exponential growth (Turnbull, 1998). For *B. anthracis*, the spore is considered the infectious particle (Driks, 2009). Spores are extremely resistant to heat, desiccation, cold, pH, chemicals and irradiation allowing them to survive in the environment for decades (Mock & Fouet, 2001; Shivachandra *et al*., 2016).

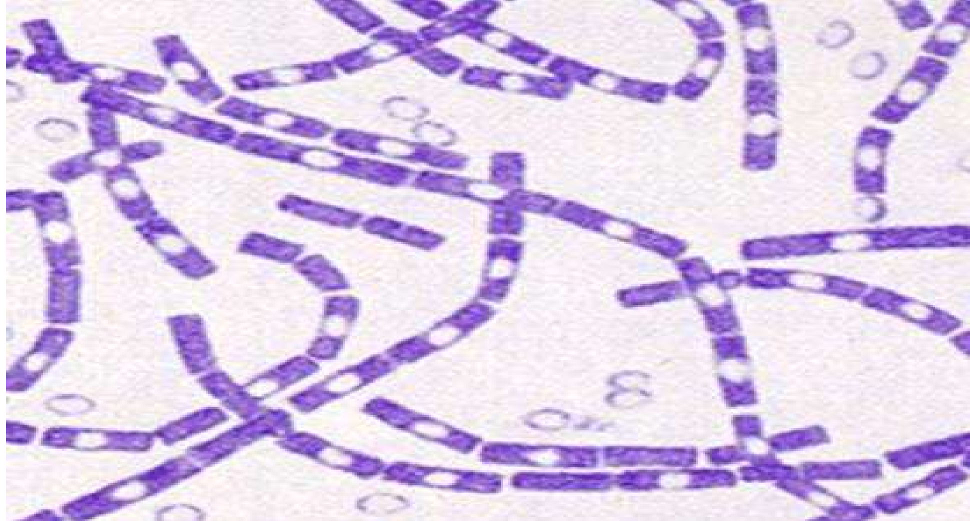

Figure 1: Gram stain result of *B. anthracis* at × 1,500 magnifications. Source: Kamal *et al*., (2011).

2.2. Epidemiology

2.2.1. Occurrences

Anthrax is a worldwide zoonosis that primary affects herbivorous animals (Spencer, 2003). In domestic animals there is high fatality in cattle, sheep, goats, donkeys and pigs (WHO, 2008). For wild animals, highest fatalities occur in zebra, antelope, bison, gazelles, impalas, elephants and hippopotami (WHO, 2008; Shivachandra *et al*., 2016). Figure 2 show worldwide distribution of anthrax outbreak per year from January 2005 to August 2016. Anthrax is the most common in agriculture region of central and South America, sub-Saharan Africa including Ethiopia, Central and southwestern Asia and southern Eastern Europe (Sweeney *et al*., 2011; CDC, 2017).

Spores survive for decades in soil that rich in calcium, has a pH greater than 6.0, and when temperature is higher than 15.5ºC (Hugh-Jones & Blackburn, 2009). Anthrax is considered as a seasonal disease as outbreaks tend to follow a prolonged hot and dry period that is followed by heavy rainfall. It is thought that heavy rainfall can carry spores during runoff in clumps of organic matter to concentrate in standing pools or puddles. It is also thought that standing water can move spores upwards into the vegetation as it dries, leaving them in a better position to be ingested by grazing animals (Hugh-Jones &

Blackburn, 2009). Vultures and wild birds are also a risk for movements of *B. anthracis* to new areas by scavenging infected animal's carcasses. It has also been shown that moving animals from endemic areas to a non-endemic area can result in the establishment of the disease in new areas, if the soil conditions are conducive to spore survival (WHO, 2008).

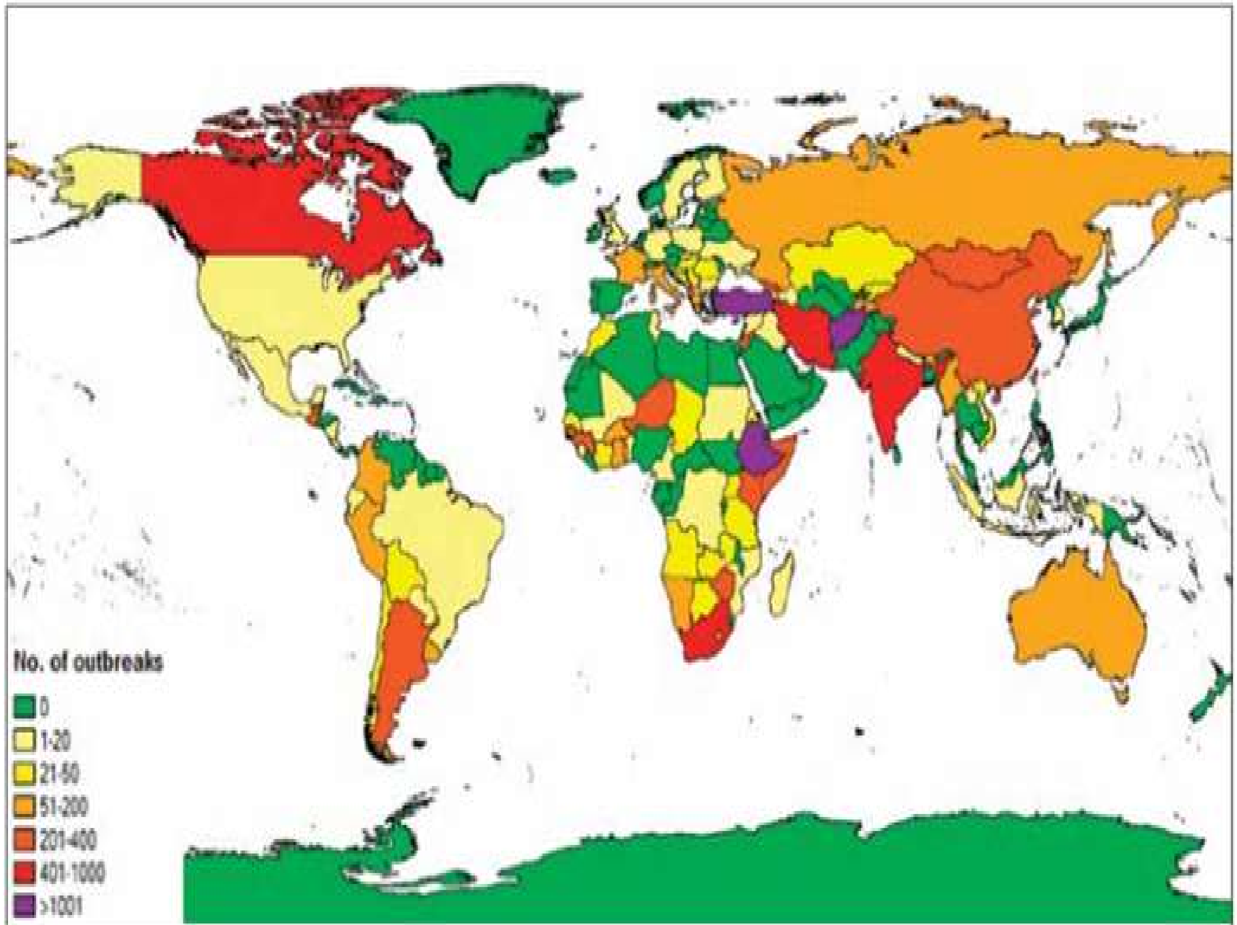

Figure 2: Worldwide anthrax outbreaks reported in livestock, wildlife and humans, January 2005 to August 2016. Source: FAO (2016).

2.2.2. Public Health Importance

Anthrax primarily affects herbivores animals. Humans usually become infected when they come into contact with infected animals or their products. Anthrax is primarily an occupational hazard for handlers of processed hides, goat hair, bone products wool and infected wildlife. It can also be contracted by contact with infected meat, for example in abattoir workers (Misgie *et al.,* 2015).

2.2.3. Economic significance

The effect of anthrax disease is a reduction of the efficiency which input or resources are converted into output or a product that means they reduced productivity. In most developing countries vaccination of susceptible animal in enzootic areas has reduced the prevalence of the disease to negligible proportions on national bases, but heavily losses may still occur in individual herds. Loss occurs due to mortality but also from withholding of milk in infected dairy herds and for a period following vaccination it also causes a great problem of death of animals, reducing animal products and complete condemnations of carcasses and by product as well as closure of abattoirs (Turn bull *et al*., 2005).

2.3. Transmissions

2.3.1. Transmission in animals

The *B. anthracis* naturally occurs in the soil, but its life cycle almost exclusively takes place within the mammalian host (Mock & Fouet, 2001). Under the right conditions, spores can survive for years in the environment. Animal hosts acquire the disease through grazing, usually by ingestion or inhalation (Mock & Fouet, 2001; Sweeney *et al*., 2011). Once enters in to the host, the spores are able to germinate back into vegetative bacilli (Figure 3). The bacilli are then able to disseminate through the host system using toxin and capsule production and once the infection becomes systemic, the host dies from shock and hemorrhages blood and other bodily liquids. After death, the vegetative cells are exposed to air causing sporulation to occur and the spores return to the soil for the next host (Mock & Fouet, 2001; Spencer, 2003; WHO, 2008, Sweeney *et al*., 2011; Moayeri *et al*., 2015).

Animal meat, hides, hair, wool or bones may be transported long distance and spores can be carried with wind currents to new areas, especially during dry period. Insects are also thought to play a role in disease transmission, primary through blow flies. Animals that

have died or are dying from anthrax provide the main source of infection for other animals through shedding of bacilli, which eventually become spores, into the environment (WHO, 2008).

2.3.2. Transmission in Humans

There are three major routs of transmission for humans: ingestion, inhalation and cutaneous. The most common form of disease is cutaneous which accounts for 95% of human cases (Sweeney *et al*., 2011; Weiner, 2012). This form is acquired through abrasions or open wounds on the skin that come into contact with either vegetative cells or spores. Cutaneous anthrax has a mortality rate of 5-20% for untreated cases (Sweeney *et al*., 2011). Gastrointestinal of anthrax develops after ingestion of spores or cells through contaminated meat. While uncommon, the mortality rate is thought to be much higher than cutaneous at 25-60%. The most fatal type of infection with anthrax is through inhalation which occurs from breathing in spores from environment (Sweeney *et al*., 2011).

Injection anthrax is becoming more common among intravenous (IV) drug user, which is characterized by cutaneous infection of soft tissue (Ramsy *et al*., 2010; Russel *et al*., 2013). This route presents differently than cutaneous infections, and has shown to be harder to treat, with infection leading to septic shock, meningitis and death in 34% patients despite treatment (Sweeney *et al*., 2011). Biting insects (Figure 3) are also thought to transmit disease, most prominently biting flies (Hugh-Jones &Blackburn, 2009; Shivachandra *et al*., 2016). Anthrax is not considered contagious, although in rare instances, human-to human transmission can occur in the cutaneous form (CDC, 2017).

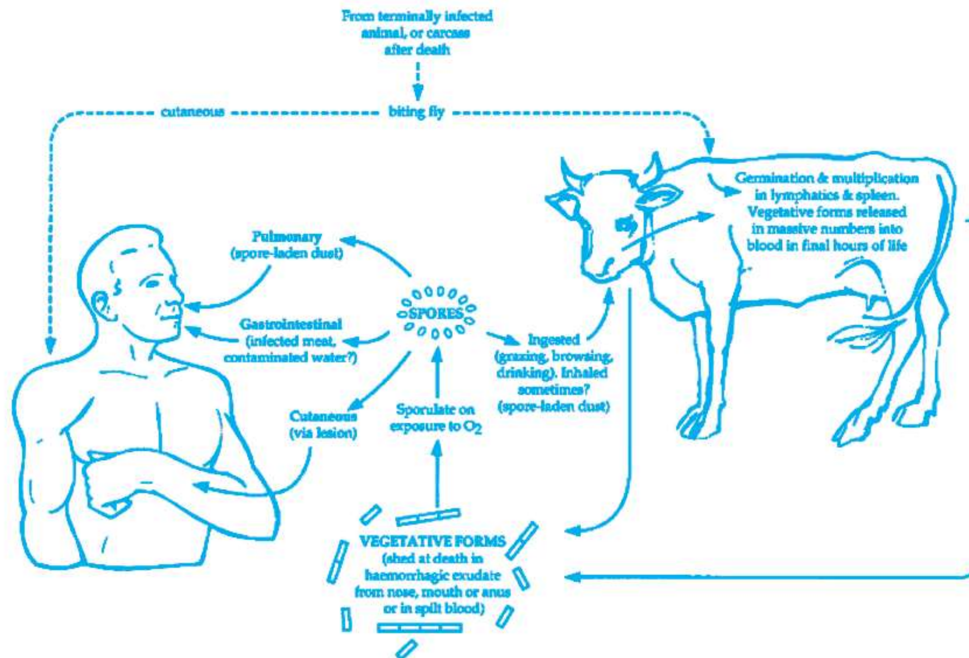

Figure 3: Transmission cycle of anthrax infection in animals and humans. Source: WHO (2008).

2.4. Clinical Progressions

2.4.1. Clinical Progression in animals

In animals, the incubation period can range from as little as 36-72 hours following the entry of the spore to 1-14 days (WHO, 2008). The typical incubation period in livestock is 3-7 days. According to the OIE international trade regulation the incubation period is considered to be 20 days. Once in blood stream, cells can have a double time as fast as 0.75-2 hours depending on the host (Shivachandra *et al*., 2016). Clinical presentation and susceptibility depends on the animal species affected.

Clinical manifestations vary from species to species, presumably reflecting differences in susceptibility. Sudden death in apparently healthy animals which may be accompanied by bloody discharges from natural orifices, rapid bloating of the carcass, incomplete rigor mortis and the absence of clotting of the blood are the common characteristics of anthrax in susceptible animals (Figure 4). In more resistant species, local signs such as swellings

of the oral and pharyngeal region are seen. In wildlife, sudden death is the invariable sign, often (but not always) with bloody discharges from natural orifices, bloating, incomplete rigor mortis and the absence of clotting of the blood (WHO, 2008).

Figure 4: A cow that has died from anthrax (Left) and A sheep that has died (Right). Note blood dripping from eyes, nostrils and mouth. Anthrax (VFF, 2017).

Horses have hyper acute to acute disease with signs for 2-3 days before death. Signs may include fever, chills, severe colic, anorexia, depression, weakness, bloody diarrhea, and swellings of the neck, sternum, lower abdomen, and external genitalia. Death usually occurs within 2–3 days of onset. Sometimes, sick horses may live up to a week. Pigs and carnivores are more resistant and may have subclinical disease. Scavengers are relatively resistant. In wild animals, local edema and swelling of the face and neck are noticed. Ultimately the animal dies of septicemia, toxemia, coma and shock. While not proven, studies have indicated that anthrax is slightly biased to older, male animals (WHO, 2008).

2.4.2. Clinical Progression in Humans

Cutaneous anthrax presents in as little as 12 hours to as long as 19 days after initial infection (Spencer, 2003), but is typically 1-12 days (Wenner & Kenner, 2004; Sweeney *et al*., 2011). A small, painless skin lesion with swelling usually appears on exposed regions of the body such as the face, neck, arms or hands (Spencer, 2003, Sweeney *et al*., 2011; CDC, 2017). The lesion eventually dries and forms a black center, called an eschar which sloughs in 2-3 weeks (Spencer, 2003; Sweeney *et al*., 2011). During this period,

fever can occur but is rare. In a small percentage of cases, septicemia can develop (Spencer, 2003). It should be noted that toxin can be detected in the bloodstream even when systemic disease is not present (Boyer *et al*., 2011).

Gastrointestinal anthrax has an incubation period of about 2-5 days and can present in two different ways: Intestinal and oropharyngeal (Spencer, 2003; Sweeney *et al*., 2011). During anthrax, vegetative cells cause ulcers or lesions throughout the small intestine (Sweeney *et al*., 2011). Symptoms include nausea, vomiting, anorexia and fever (CDC, 2017). In severe cases, abdominal pain, hematemesis, bloody diarrhea and septicemia can present. Oropharyngeal anthrax occurs when vegetative cells settle in the pharyngeal area and produce ulcers. Patients usually present with fever, neck swelling and sore throat (Spencer, 2003; CDC, 2017).

Inhalation anthrax (also known as pulmonary) has the highest mortality and has historically been linked to industrial cases of disease, although bioterrorism has become the greatest concern in large-scale outbreaks (Sweeney *et al*., 2011; Weiner & Glomski, 2012). Inhalation cases take a biphasic clinical course (Sweeney *et al*., 2011). After approximately four days, patients develop flu-like symptoms with fever, cough and myalgias (Sweeney *et al*., 2011). During the second phase, patients presents with high fever, tachyacardia, moistrales, hypotension and dyspnea. After these symptoms, the patients typically become disoriented, which progresses to a coma and death within 24 hours of onset as reported (Spencer, 2003; Sweeney *et al*., 2011).

2.5. Pathogenesis

The main virulent factor are encoded in plasmid. The plasmid are circular, extra chromosomal, double stranded DNA molecule (Read, 2003). Infection occurs after introduction of the spore through a break in the skin (cutaneous anthrax) or entry through mucosa (gastrointestinal anthrax). After ingestion by macrophages at the site of entry, germination of the vegetative form then occurs, followed by extracellular multiplication, together with the production of capsule and toxins. The capsule plays an important role in

establishment of the infection while the toxins are more prominent in the final stages of infection (Moayeri *et al*., 2015).

2.5.1. Toxins

Genes encoding toxins (pX01) includes genes for both lethal toxin and edema toxin while pX02 includes genes for production and assembly of the capsule (Mock & Fouet, 2001; Spencer, 2003; Sweeney, 2011; Moayeri *et al*., 2015). The toxin complex is composed of three proteins: lethal factor (LF), edema factor (EF) and protective antigen (PA). PA is an intra-member transporter that mediates cell binding and uptake of LF and EF (Sweeney *et al*., 2011). Protective antigen in combination with EF and LF forms edema toxin and lethal toxin, respectively (Mock & Fouet, 2001).

Edema toxin alters the production of cyclic-Adenosine mono phosphate (C-AMP) which creates altered ions and water movements. This leads to the characteristic edema of anthrax. It is also thought to impair neutrophil function and prevent the inflammatory process (Shivachandra *et al*., 2016). Lethal toxin is an endopeptidese that disrupts signaling pathways and leads to the synthesis of cytokines that ultimately cause septic shock. It is also thought that lethal toxin may attack the endothelial cell linings of the capillary network that results in the necrosis of blood vessels. This leads to the characteristic hemorrhage from the nose, mouth and anus of infected hosts and the systematic release of bacilli into the environment, completing its life cycle (Shivachandra *et al*., 2016).

2.5.2. Capsule

The capsule is a polymer that encases the bacterium. The capsule of *B.anthracis* is unique in that it is made from the protein, poly-glutamic acid, and not carbohydrate. It is formed under elevated CO_2 condition and in the presence of bicarbonate. The capsule allows virulent bacilli to grow unimpeded in the host for the initial stages of infection. It is

theorized that the negative charge of the capsule inhibits host defense mechanisms, namely phagocytosis, allowing the bacteria to establish an infection (Zwartouw & Smith, 1955; Makino *et al*., 1989; Ezzell & Welkos, 1999). The smaller capsule bearing plasmid pXO2 is 95.3 kbp in size and encodes three genes (*cap B, cap C*, and *cap A*) involved in the synthesis of the poly-glutamyl capsule that inhibits host phagocytosis of the vegetative form of *B. anthracis* (Makino *et al.*, 1989).

2.6. Spore and vegetative cell survival

Vegetative cells in unopened carcasses may survive for up to 1 to 2 weeks, but spores can persist for decades in a stable, dry environment. Spores are killed by autoclaving (121ºC/15min) and dry heat (150ºC/60 min), but not by boiling (100ºC) for under 10 minutes. They are not highly susceptible to phenolic, alcoholic, oxidizing and chlorinating disinfectants, beta-propiolactone, and ethylene oxide are more useful. Heat fixation of smears does not kill spores (Hirsh & Yuan, 1999).

2.7. Diagnosis

Suspicion of anthrax was arising from the observation of clinical symptoms, pathological lesions and epidemiology of the disease. Clinical manifestations vary from species to species, presumably reflecting differences in susceptibility. Sudden death in apparently healthy (domestic and wild) animals which may be accompanied by bloody discharges from natural orifices, rapid bloating of the carcass, incomplete rigor mortis and the absence of clotting of the blood was the characteristics of anthrax in susceptible animals. In more resistant species, local signs such as swellings of the oral and pharyngeal region are seen (WHO, 2008).

Based on CDC recommendations, a confirmed anthrax case is defined as a clinically compatible one with isolation of *B. anthracis* or with at least two positive supportive tests using serologic or other methods (CDC, 2001).

2.7.1. Biosafety Procedures

Before specimen collection, proper personal protective equipment (PPE) should be worn and any existing cuts abrasions should be dressed. PPE depends on the type of specimen being sampled. Fresh specimens require a laboratory coat/gown and apron with disposable covers for feet. Double gloving is recommended as the outer glove can be changed easily without exposing the skin to any hazardous material. This also helps prevent cross contamination of other animals, if sampling is necessary for more than one carcass. If working with old specimens such as hides or bones that have the potential to create dust, respiratory protection (Such as N95 respirators) is also recommended. After specimen collection, rinse or wipe down gloved hands with 0.5% hypochlorite solution and discard outer gloves. Discard used PPE into proper disposal bags, separating them into autoclaveable and non-autoclaveable items (WHO, 2008).

Clinical specimens and cultures of *B. anthracis* should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (OIE, 2018). If the samples are not being processed in a safety cabinet, protective eye-shields and good-quality face masks may be advisable to protect the operator from other (non-anthrax) infectious agents that might be present; availability of high-quality, properly positioned facilities for hand-washing and careful dressing of skin abrasions with the appropriate safety precautions. Old dried-up specimens, such as old hides, that are liable to give off dust during processing, should be handled in a biosafety cabinet, preferably class 3 (WHO, 2008).

2.7.2. Laboratory diagnosis

Good laboratory diagnosis of anthrax starts from proper sampling from animals that depends on the type of specimen being examined. Specimens can be from untreated animals, from old and decomposed animal carcasses/product (Table 1) or from environmental samples (WHO, 2008). It needs to be kept in mind that carcass should never be opened to perform a necropsy of suspected animal died of the case.

Table 1: Appropriate specimen and collection methods for animals suspected of anthrax.

Circumstance	Specimen	Container
Fresh carcass	Poolded blood(0.1ml), blood and fluid from body cavity if opened (by scavengers), ear clipping, or swab	Small vial, or leave in syringe, or fluid collected on a swab.
Putrefied carcass	Piece of highly vascularized tissue and swabs of nasal turbinates, eye socket, any bloody materials. Visible bloody soil from under head or tail.	Swabs with tubes. For soil, sealable specimens container.
Very old carcass, hides, bones, soil around/under carcass	Swabs of nasal turbinates, eye socket. Soil from where body fluid believed to have fallen.	Swabs with tubes. For soil, sealable specimens container.

Source: WHO (2008).

2.7.3. Criteria for laboratory diagnosis of anthrax

In suspected laboratory diagnosis, the smear shows Gram-positive, square-ended rods in pairs or short chains, occasionally singly, in association with a suggestive clinical history. Presumptive laboratory diagnosis includes smear stained with poly-chrome methylene blue shows dark blue square-ended rods in pairs or short chains, occasionally singly, surrounded by pink capsule. A confirmatory laboratory diagnosis test shows culture of *B. anthracis* and confirms toxin and capsule genes by PCR (WHO, 2008).

2.7.4. Culture and Gram Stain

Bacterial culture and isolation is considered the gold standard and most important diagnostic tool for identification of *B. anthracis* and easy to grow on nutrient agar medium (Shadomy & Smith, 2008). Specimen or culture can be grown overnight on Sheep Blood agar at 35-37ºC. However, on sheep Blood agar (5%) and other routine culture media, almost all *Bacillus* species grow well (Berg *et al*., 2006). After incubation for 18–24 hours, growth occurs on blood agar and shows the characteristic morphology of grey/white, flat colonies, 2–5 mm in diameter, Flat or slightly raised, gray to white with a "ground glass" appearance and described as "tenacious" or "sticky" like petroleum jelly, (Figure 5). After 18 hours of incubation on sheep blood agar (SBA) at 35°C, the slightly undulate margin may show curling, displaying a so-called "Medusa head" or described as comma-shaped protrusions (CDC, 2017). Colonies should be observed for hemolysis (the rupture of red blood cells resulting in clearing of the medium) after incubation and a Gram stain should also be performed. *B. anthracis* will be negative for hemolysis and appear as purple rods after Gram stain (Mathias & Todd Parker, 2009).

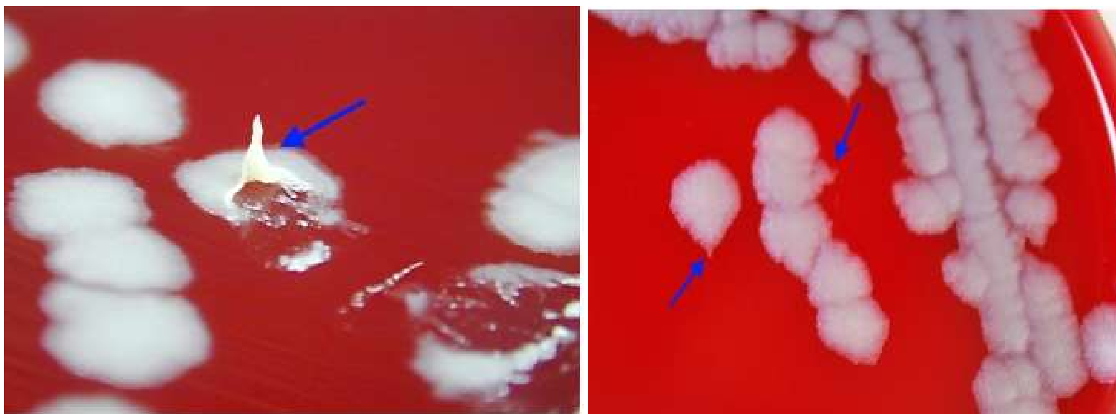

Figure 5: Colony morphology of *B. anthracis* grown overnight on blood agar. Source: CDC (2018).

A selective media containing polymyxin-B, lysozyme, EDTA and thallous acetate (PLET media) can also be used for isolation of *B. anthracis* from contaminated and suspected samples (Marston *et al*., 2008**)**. It consists heart infusion agar with polymyxin, lysozyme,

ethylene diamine tetra acetic acid (EDTA) and thallous acetate (pH 7.35). Commercial readymade PLET agar base is also available to be used with Anthracis Selective Supplement (FD185) (Knisely, 1966). Another media (bicarbonate agar) is used to induce capsule formation for subsequent identification of *B. anthracis*. However, there is very little utility of these selective growth media because several closely related bacteria of *B. anthracis* like *B. cereus* and *B. subtilis* also grow well on these media.

2.7.5. Biochemical Test

B. anthracis is highly susceptible to penicillin and *B. cereus* and the other spp are resistant. *B. anthracis* slowly produces an inverted fir tree type of gelatin liquefaction with side-shoots radiating from the stab line but *B. cereus, B. mycoides, B. thuringiensis* rapidly liquefy nutrient gelatin and non motile (Table 2). *B. anthracis* characterized by various biochemical tests like catalase, oxidase, nitrate reduction, hemolysis, citrate utilization, urease (Cheesbrough, 1985).

Table 2: Differential characteristics of *B. anthracis* and *B. cereus*.

	B. anthracis	*B. cereus*
Hemolysis	-	+
Motility	-	+
Lysis by gamma phage	+	-
Capsule production	+	-
Penicillin susceptibility (10 unit disc)	S	R

+: positive reaction; -: negative reaction; S- Susceptible; R: Resistant; Source: Quinn *et al*. (1994).

Capsule production: Samples can also be tested for capsule production by incubation on certain types of agar followed by staining. Single colonies from Sheep Blood agar can be

inoculated on to three different types of media. The first is Heart infusion broth (HIB) with 0.8% sodium bicarbonate. The broth should be incubated at 35-37ºc for 6-8 hours. Incubation can happen at an ambient atmosphere in a Co_2 enriched environment. The characteristic mucoid or smooth colony variant is correlated with capsule production ability of *B. anthracis* on capsule agar incubated in an atmosphere of 5% CO_2. The second medium is defibrinated horse blood. This should be incubated at 35-37ºC for 6-8 hours in an ambient atmosphere. The third medium is HIB supplemented with heat-inactivated horse serum and 0.8% sodium bicarbonate. This broth should be incubated at 35-37ºC for 6-8 hours in an ambient atmosphere or in a CO_2-enriched environment (WHO, 2008).

M'Fadyean (Polychrome methylene blue) is a simple stain containing methylene blue that is applied to fixed smears to visualize capsule. After staining, bacilli will appear dark blue with a narrow area around and between that is red/purple (Figure 6). A sample of culture should be smeared on a microscope slide and allowed to dry and then be heat-fixed. Stain is added to the smear and rinsed. The slide can be viewed at 40x or 100x with oil immersion (Todar, 2012). M'Fadyean-stained blood smears examined at death will reveal large numbers of the capsulated bacilli which can also be isolated and confirmed bacteriological (WHO, 2008).

Indian Ink stain will stain surrounding cells but will not stain the capsule, if it is present. This will result in a halo or glow around cells that contain capsule. A mixture of culture and Indian ink should be viewed on a microscope slide at 40x or 100x with oil immersion.

Figure 6: Left M’Fadyean Stain (Todar, 2012) and Right capsule stain with Indian ink (Shauffer, 2002).

Gamma-phage Lysis: is a bacterial virus that specifically lyses *B. anthracis* with 96% specificity. Most other strains of the *B. cereus* groups are not susceptible to lysis by Gamma-phage. A single colony is spread onto Sheep Blood agar plate, and small amount of gamma phage is aliquoted onto the first or second quadrant. If the test organism is susceptible to the phage, a clear zone of lysis will be present after overnight incubation at 35-37°C (Figure 7). While gamma-phage lysis is a simple test to perform, proper propagation of active gamma-phage is essential. Unstable phage preparation can lead to false negative results (WHO, 2008).

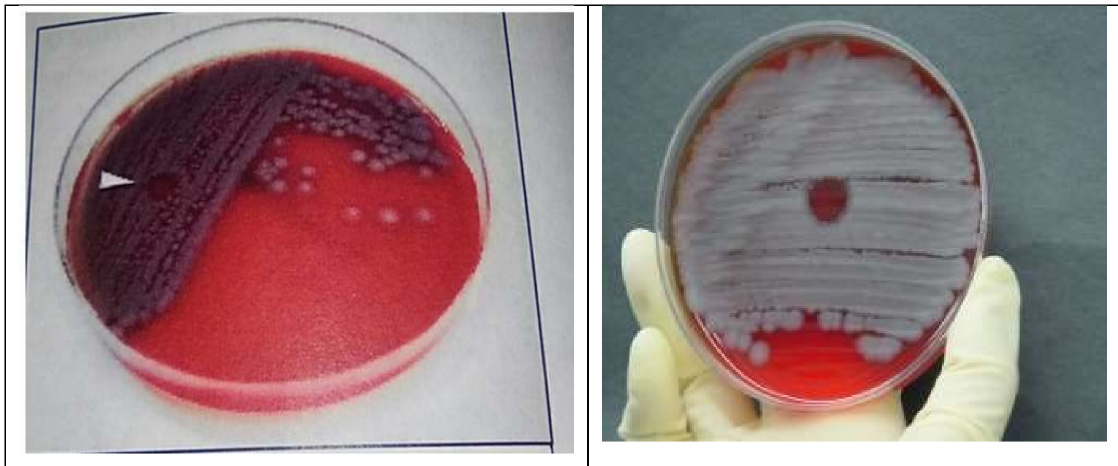

Figure 7**:** Gamma-phage assay with positive results. Source: Mathias & Todd Parker (2009).

2.7.6. Serological Tests

Enzyme-Linked Immunosorbent Assay (ELISA) combines the specificity of antibodies with the sensitivity of simple enzyme assays. In very simple terms, a small amount of sample is affixed to surface and is then covered with a mixture of antibody that is linked to an enzyme. If the antigen for this antibody is present, it will bind to the affixed sample. In the final step, a substance is added that the enzyme can convert to some detectable signal. This usually results in a color change, giving a detectable signal that the antigen is present in the sample. For anthrax, protective antigen is the target used for diagnosis. A serum sample should be collected during the acute and the convalescent phase of illness. A sample is considered positive if there is a > 4-fold increase in the amount of PA antibodies from the acute to the convalescent serum (Quinn *et al*., 1994).

The presence of anti-PA IgG in human serum can be an accurate indicator of anthrax exposure. Simultaneously, it can confirm the efficacy of vaccine in humans as well as in animals. Earlier reports also showed that anti-PA IgG enzyme-linked immunosorbent assay (ELISA) is a valuable tool for confirmation of cutaneous and inhalational anthrax cases (Quinn *et al*., 1994). In a recent study, anti-PA IgG and anthrax lethal toxin neutralization activity levels were found to be very useful for detection and confirmation of natural cutaneous anthrax cases in Bangladesh (Boyer *et al*., 2011).

Ascoli Test is to supply rapid retrospective evidence of anthrax infection in an animal. It was designed to detect *B. anthracis* antigens in the tissues of animals being utilized in animal by-products, and thereby to reveal when these products contained ingredients originating from animals that had died of anthrax (WHO, 2008). This thermo precipitation test is used if viable *B. anthracis* can no longer be demonstrated in tissues. About 2-3 gm of homogenized materials in a little saline is briefly boiled and passed through filter paper. This filtrate is used as the antigen in a ring precipitation or gel diffusion test with known *B. anthracis* precipitating antiserum and the test is not suitable for detection of *B. anthracis* in environmental specimens (Quinn *et al*., 1994).

2.7.7. MALDI-TOF Mass Spectrometry

Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MADLDI-TOF MS) can be utilized to detect lethal factor in serum samples of patients in the acute stage of illness. MADLDI-TOF MS is a molecular technique used to separate protein or peptides of samples, seen as peaks on a spectrum. By analyzing serum samples mixed with a peptide that can be cleaved by lethal factor (LF), the presence of LF in the sample can be determined. If is not present, a single protein peak will be seen after MADLDI-TOF MS (Figure 8) analysis while if LF is present, two distinct peaks will be seen as a result of the cleavage. This is only applicable to acute samples, as LF is circulating in the blood at that time (Boyer *et al*., 2007).

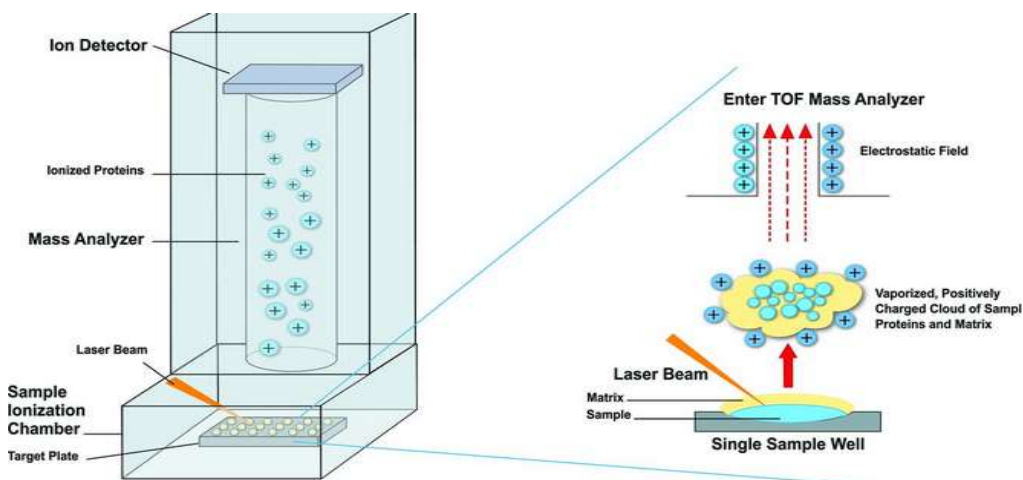

Figure 8: MALDI-TOF mass spectrometer. Source: Patel (2013).

2.7.8. Molecular diagnosis

Conventional PCR is standard PCR reactions contained 2.5 U of Taq polymerase, 300 nM of each primer, all four dNTPs at 200 nM each and 2 mM MgCl2. Purified DNA and water were added to a total volume of 50 µl. PCR reaction was performed in a 9600 thermal cycler with 2 min of denaturation at 94°C and 33 cycles with 20 second at 94°C,

20 second at 60ºC and 30 second at 72ºC. Amplicons were analyzed by conventional DNA electrophoresis in a 2% (Ellerbrok *et al*., 2002).

For conventional PCR sodium dodecylsulfate (SDS) used lysates from *B. anthracis* colonies incubated for 30 min at 80^{0}C in SDS containing ATL buffer (Qiagen tissue kit). Lysates were centrifuged in order to eliminate or reduce the number of spores. It was observed by plating that in some of the preparations residual viable spores were present. In addition, these lysates had to be pre-diluted at least 100-fold to avoid interference of SDS with the PCR reaction, thus reducing the sensitivity of the assay without completely solving the safety problems (Ellerbrok *et al*., 2002).

Table 3: Suitable primers for confirming the presence of the pXO1 and pXO2 plasmids

Target	**Primer ID**	**Primer Sequence 5'-3'**	**Product size**	**Concentration**
Protective antigen (PA)	PA 5 3048-3029	TCC-TAA-CAC-TAA-CGA-AGT-CG	596 bp	1mM
	PA 8 2452-2471	GAG-GTA-GAA-GGA-TAT-ACG-GT		
Capsule	123 11411-1430	CTG-AGC-CAT-TAA-TCG-ATA-TG	846 bP	0.2mM
	1301 2257-2238	TCC-CAC-TAA-CGT-ATT-CTG-AG		

Source: OIE (2018).

Real-Time Polymerase Chain Reaction (PCR) is molecular techniques used for detection of *B. anthracis*. The most common use of real-time PCR in anthrax is for diagnostic purpose. *B. anthracis* phylogenetically belongs to the *B. cereus* group. Members of this group share similar chromosomes but differ greatly in their extra chromosomal genetic elements which accounts for significant difference in phenotypic properties, such as the production of virulence factors. Loss of either factors results in a significant decrease in virulence (Moayeri *et al*., 2015).

Primers to one of the toxin genes (usually protective antigen) and to one of the capsule genes are used to confirm the presence of virulent *B. anthracis* (WHO, 2008). However, there is evidence that other members of the *B. cereus* group can produce *B. anthracis* like plasmids (Hoffmaster *et al*., 2006; Moayeri *et al*., 2015). These *B. cereus* strains have been shown to cause anthrax-like disease in animals, and have been isolated in several countries including the United State, Cameroon, Cote d'Ivoire (Hoffmaster *et al*., 2004; Klee *et al*., 2006). These strains harbor a plasmid pBCXO1 that, similar to pXO1, that has genes for toxin production (Hoffermaster *et al*., 2006). They also produce a capsule, although it is not encoded on a plasmid as in *B. anthracis* (Hoffmaster *et al*., 2004), and also carry some genes very similar to those on pXO2 on anther plasmid (pBCXO2) (Antonation *et al*., 2016).

Due to the presence of these factors, *B. cereus biovar anthracis* can cause positive results using anthrax real-time PCR methods. These isolates differed significantly from classic *B. anthracis* by the following criteria: motility, resistance to the gamma phage, and, for isolates from Cameroon, resistance to penicillin G. A capsule was expressed not only after induction by CO_2 and bicarbonate but also under normal growth conditions. However, there have been no known cases of human infection caused by *B. cereus biovar anthracis* to date (Klee *et al*., 2006).

Real-Time PCR can also be used to collect data for epidemiological surveillance and prevention programs. Genetic analysis of environmental samples taken from enzootic areas can determine where anthrax is naturally found and where animals are at the highest risk infections (WHO, 2008).

B. anthracis, the highly dangerous zoonotic bacterial pathogen species is currently composed of three genetic groups, called A, B and C. Group A is represented worldwide whereas group B is present essentially in Western Europe and Southern Africa. Only three strains from group C have been reported (Vergnaud *et al*., 2016). Multilocus sequence typing (MLST) is considered the best methods. Multi-locus variable-number

tandem repeat analysis and sequencing of genes coding for 16S ribosomal RNA may be conducted for species identification and molecular characterization of *B. anthracis* isolates (Sweeney *et al*., 2011).

Whole genome sequencing (WGS):- is modern molecular strain typing techniques now becoming available are beginning to make it possible to distinguish outbreaks caused by different strains from those caused by the spread of a single strain, to trace an outbreak strain back to its possible origin and to track the routes of transmission of an outbreak strain within and between animal populations (Keim *et al*., 2000).

The complete sequencing and annotation of the 181.7-kb *Bacillus anthracis* virulence plasmid pXO1 predicted143 genes but could only assign putative functions to 45. Hybridization assays, PCR amplification, and DNA sequencing used to determine whether pXO1 open reading frame (ORF) sequences were present in other bacilli and more distantly related bacterial genera. ORFs were conserved in most of the bacteria. Many of the pXO1 ORFs were detected in closely related *Bacillus* species, and some were detected only in *B. anthracis* isolates. The majority of the DNA fragments that were amplified by PCR had DNA sequences between 80 and 98% similar to that of pXO1. Pulsed-field gel electrophoresis also revealed large potential plasmids present in both *B. cereus* 43881 (341 kb) and *B. thuringiensis* ATCC 33679 (327 kb) that hybridized with a DNA probe composed of six pXO1 ORFs (Pannucci *et al*., 2001).

Plasmid pXO2 (60 MDa) carries genes required for the synthesis of an antiphagocytic poly-D-glutamic acid capsule (Green *et al*., 1985; Uchida *et al*., 1993). The 110-MDa plasmid pXO1 (61) is required for synthesis of the anthrax toxin proteins, edema factor, lethal factor, and protective antigen. These proteins act in binary combinations to produce the two anthrax toxins: edema toxin (a protective antigen and edema factor) and lethal toxin (a protective antigen and lethal factor). Plasmid pXO2 carries three genes required for capsule synthesis (*capB*, *capC*, and *capA*), a gene associated with capsule degradation (*dep*), and a *trans*-acting regulatory gene (*acpA*) (Green *et al*., 1985; Uchida *et al*., 1993).

2.7.9. Differential diagnosis

Anthrax should be differentiated from other causes of sudden death such as: lightning strike and accidental electrocutions, pasteurellosis, piroplasmosis, blackleg, malignant oedema, food intoxications, botulism, peracute babesiosis, chemical poisoning (heavy metal and other poisoning), plant poisoning, snake bite, metabolic disorders (lactic acidosis), magnesium deficiency, bloat and others (Hugh-Jones & de Vos, 2002).

2.8. Anthrax Situation in Ethiopia

2.8.1. *Reported outbreaks of Anthrax in Ethiopia*

Anthrax is an endemic disease which occurs in May and June every year ('anthrax season') in several farming localities of the country, causing disease both in domestic and wild animals and humans. It is still a significant risk in most regions in Ethiopia and outbreaks frequently occur in humans and animals (Shiferaw, 2004).

In the country a retrospective record review from 2009-2013 showed that within five years a total of 26737 animal cases with 8523 animal deaths due to anthrax were reported (Bahiru *et al*., 2016). This data showed that each year on average death of 1705 animals were recorded due to anthrax. Based on the report of Shiferaw (2004), 26 cases of anthrax in animals were reported in Wabessa village of Dessie Zuria district, as a result death of 26 animals were recorded from the outbreak.

Moreover, Retrospective study on the epidemiology of bovine anthrax in Elu Aba Bor Zone, South West Ethiopia showed that from the period of 2009-2016 within 8 years' duration a total of 405 anthrax outbreaks with 1166 case and 739 deaths in cattle were registered (Table 4). This data revealed that each year 50 outbreaks of anthrax occurred in the area. Based on the report the hot dry season accounted for 29.6% of the outbreaks followed by the rainy and cold dry season (24.69%), and the post rainy season recorded the lowest proportion 20.99% (Eshete *et al*., 2017).

In wild life anthrax outbreak has been reported in southern parts of Mago national parks and spread to the northern part within two months beginning from September 1999. Moreover, another anthrax outbreak from September to October 2000 has also been reported in this park. According to the report in the first outbreak more than 1,600 wild animals were dead from 21 different species. Of all the species *Lesser kudu* was severely affected, which accounts for 95% of mortality and as a result more than 65% of *Lesser Kudu's* population within the park died (Shiferaw *et al*., 2002).

The national retrospective data showed that a total of 5,197 human anthrax cases were reported from 2009 to 2013 with 86 human anthrax deaths (Case Fatality Rate: 1.7 %). The national human prevalence was found to be 1.3 per 100,000 populations per five years (Bahiru *et al*., 2016). This report revealed that each year on average 17 people death was recorded due to anthrax in the country. Based on the report from Shiferaw (2004) 6 human cases and 3 human deaths were recorded due to anthrax in Dessie Zuria district of Amhara Regional state. Gelaw and Asaminew (2013) reported a case series of 3 patients with periocular anthrax that were seen at Jimma University Specialized Hospital, Ethiopia from June 2011 to May 2012. From the report all the three patients responded to intravenous antibiotics, and the lesion resolved leaving scars which caused cicatricial ectropion in all cases.

The study reported by Perez-Tanoira *et al*. (2017) described the main clinical characteristics of cutaneous anthrax in eight patients (six females and two males, age range 1 – 56 years) admitted to the rural General Hospital of Gambo, West Arsi Province of Ethiopia from 2010–2013. According to the report patients responded positively to treatment, and the lesions resolved, leaving eschars. However, one patient suffered the loss of an eyeball, and another died 12 hours after starting treatment. The study recommended that physicians working in rural areas of resource-poor settings should be trained in the clinical identification of cutaneous anthrax. Early antibiotic treatment is essential for decreasing morbidity and mortality. Moreover, Ethiopian weekly Epidemiological Bulletin of Ethiopian Public Health Institute reported 38 human cases

and 1 death due to anthrax from different part of the country within one week in May 2018 (EPHI, 2018).

Table 4: Animal and human anthrax cases and deaths reported in different parts of Ethiopia.

Year	Animal		Human		References
	Cases	Death	Cases	Death	
2018	NR*	NR*	38	1	EPHI, 2018
2009-2016	1166	739	NR*	NR*	Eshete *et al*., 2017.
2010–2013	NR*	NR*	8	1	Perez-Tanoira *et al*., 2017
2009-2013	26737	8523	5197	86	Bahiru *et al*., 2016
2011-2012	NR*	NR*	3	0	Gelaw and Asaminew, 2013
2002	26	26	6	3	Shiferaw, 2004
Total	27,929	9,288	5,250	91	

* NR-Not reported

2.8.2. *Laboratory diagnostic capacity of anthrax in Ethiopia*

The country has established laboratory system with Regional laboratories working in collaboration with the National referral laboratories in both public and animal health sectors. To date in both public and animal health sector, outbreak reports of anthrax are based on history and clinical signs and there are limited laboratory confirmed cases at either the regional or national level. National and regional laboratories experience for the diagnosis of anthrax is mostly limited to polychrome methylene blue staining techniques (M'Fadyean stain), that is through staining blood smears from peripheral blood vessels and observing the square ended, blue rods in short chains surrounded by pink capsule through direct microscopy.

Moreover, other staining techniques like Gram staining and Giemsa staining techniques are also practiced. But the experience of conducting gold standard tests for laboratory

confirmation of anthrax using culture and PCR is limited. In addition to limitations in confirming anthrax, biosafety and biosecurity issues are also of major concern for culture and identification of *B. anthracis* at the regional laboratories. Currently the National reference laboratories; Ethiopian Public Health Institute and National Animal Health Diagnostic and Investigation Center have built the technical capacity to diagnose anthrax by Real time PCR method. Moreover, on one health approach national and regional public and animal health laboratory staffs were also trained on proper ways of sample collection, transportation and storage from anthrax suspected cases (EPHI, 2018).

2.9. Treatment and Preventions

The control of anthrax outbreaks among domestic animals is primarily dependent on rapid identification and treatment of affected animals; enhanced surveillance for additional cases; implementation of control measures including quarantine, prophylaxis, and vaccination; prevention of animal access to suspected sources such as potentially contaminated feed or pastures; and appropriate disposal of infected carcasses and disinfection of affected premises.

The recommended procedure for treating animals showing clinical illness in which anthrax is thought to be the likely or possible cause is immediate intravenous administration of sodium benzylpenicillin as directed by the manufacturer's instructions (usually in the range 12 000–22 000 units per kg of body weight) followed 6–8 hours later by intramuscular injection of long-acting benethamine penicillin (manufacturers' instructions usually recommend a dose within the range of 6000–12 000 units per kg of body weight) or other appropriate preparation such as Clamoxyl® (15 mg/kg), a long–acting preparation of amoxicillin (WHO, 2008).

If long-acting preparations are unavailable, procaine penicillin (the dose recommended by manufacturers is usually 6000–12 000 units/kg) can be used for intramuscular injection, but should be administered again after 24 and 48 hours. Recommended doses of streptomycin to be administered together with penicillin intramuscularly are 5–10 mg

per kg body weight in large animals and 25–100 mg per kg body weight in small animals (WHO, 2008).

B. anthracis is susceptible to numerous antibiotics but treatment must begin early enough in infection to be successful. Penicillin and ciprofloxacin are considered the drug of choice although tetracycline, chloramphenicol, aminoglycosides, macrolides, imipenem, rifampicin and vancomycin can be used commonly in human (Spencer, 2003; Moayerl, 2015; CDC, 2017). Antibiotic treatment usually continues for 7-10 days although it can be extended in severe cases (Spencer, 2003). Along with antibiotics treatment, supportive care may be required as fluid drainage, blood pressure support and mechanical assistance with breathing (WHO, 2008).

Vaccination of host animals particularly cattle, sheep and horses, remains the gold standard for prevention of anthrax among both animals and people (Shivachandra, 2016). The veterinary anthrax vaccine is a live strain that contain the pXO1 plasmid but is missing the pXO2, meaning it is toxinogenic but non-encapsulated (Mock, 2001).

2.9.1. Availability of Anthrax vaccine in Ethiopia

Anthrax livestock vaccine is being produced at National Veterinary Institute (NVI) for the last 50 years. NVI is the sole supplier of the vaccine in Ethiopia and the availability of the vaccine provides favorable opportunity in the control and prevention of Anthrax in Ethiopia.

The vaccine is prepared from live spores of a non-encapsulated but toxigenic ($pXO1^{+}/pXO2^{-}$) variant of *B. anthracis* 34F2 Sterne strain isolated in 1937 by Sterne. This seed strain is used worldwide for animal anthrax vaccine production since it is stable and has lost virulence, but is still immunogenic, due to its incapability of forming a capsule, a characteristic coded by the pXO2 plasmid. The vaccine produced is certified for its safety and protective efficacy.

The vaccine produced at NVI is composed of *B. anthracis* (Sterne strain) containing10^7 spores/dose, saponine (0.1%), stabilizer (4% skimmed milk). The vaccine is indicated for use in cattle and sheep but can also be used for all susceptible domestic animals which are at risk of acquiring anthrax due to contact with the contaminated soil, forages, or carcasses of infected animals. The vaccine is administered subcutaneously or intradermal at a dose of 1ml for cattle and horse, 0.5ml for sheep and goat and can effectively confer protection against anthrax for a year. The final product is available in freeze dried form and must be kept at +4ºC to -20ºC protected from light. These conditions should be maintained from production until utilization (NVI, 2017).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

This study was conducted from November 2018 to June 2019 through-out Ethiopia, where five anthrax suspected outbreaks were reported and investigated. The *firs*t anthrax suspected outbreak was reported from Darimu Dstrict in March, 2019. The area is located in Illubabor Zone of Oromia Regional state, which is located 660 kms away from Addis Ababa, west of Ethiopia and Darimu Districts, Enariyo Kebele which is located at 8°.37123 N latitude, 35°.26079 E longitude and 1640 meters above sea level.

The *second* anthrax suspected outbreak was from Amhara regional state, South Gonder Zone, Farta District, Wowu Kebele in April, 2019. The area is located 11°.9300 N latitude, 38°.0917 E longitude and 2606 meters above sea level (masl).

The *third* outbreak was investigated, in SNNPR, Wolayita-Sodo Zone, Damota Sore District, Gurmuledisa, Kebele in the beginning of April, 2019 and the area is located 355 km south of Addis Ababa and located at 6°.6220 N latitude, 37^{0}.6600 E, longitude and 2036 masl. Another outbreak from the same region was reported from Gurage Zone, Abeshege District, Ghibe Yemengistiersha Kebele, located at Gibe sheleko national park 180 kms south west of Addis Ababa at 8°.2336 N latitude, 37°.5708 E, longitude.

The *fifth* anthrax suspected outbreak was reported from Ewa and Awra Districts, Zone 4 of Afar regional state in three Kebeles at the end of May, 2019. Ewa District is located at 630km north east of Addis Ababa at 11°.8970 N latitude, and 40^{0}.1777 E, longitude and 880 masl and Awra District is located at 670km north east of Addis Ababa at 12°.43611 N latitude, and 40^{0}.6250 E, longitude and 850 masl.

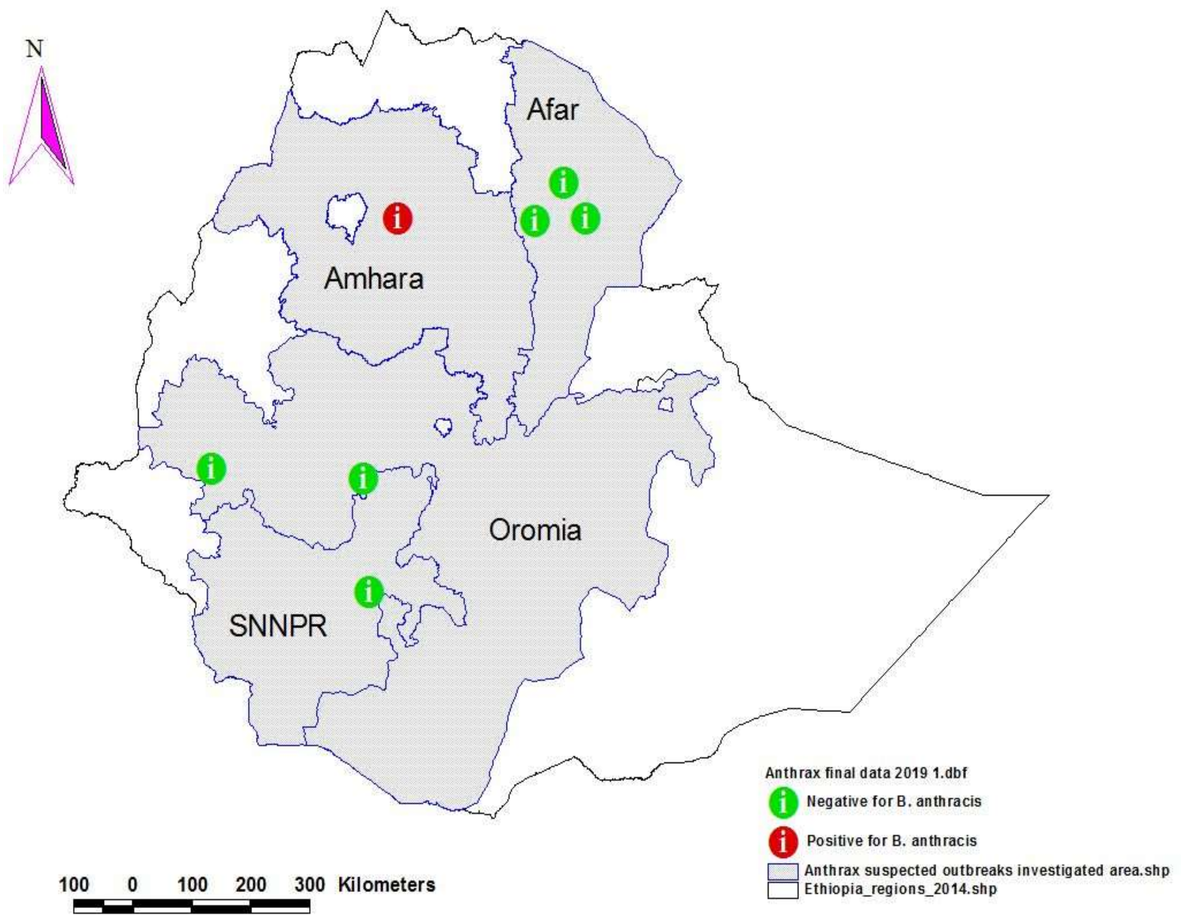

Figure 9: Map of Ethiopia showing suspected anthrax outbreak investigation areas

3.2. Study subjects

The study population was all susceptible domestic animals (particularly cattle and goat) and wild animals (Hippopotamus) where five anthrax outbreaks were investigated and a total of 63 samples were collected.

3.3. Study Design

A serious cases study design and purposive sampling methods were used during active outbreak searching to isolate, identify and detect *B. anthracis* from anthrax suspected cases. Moreover, information of outbreak was gathered from concerned bodies (outbreak investigation laboratories, district veterinary clinic workers and owners). Clinical information was recorded and appropriate samples were collected from suspected cases and the collected samples were transported to National Animal Health Diagnostic and Investigation Center (NAHDIC), for laboratory diagnosis.

3.3.1. Data Collection Method

A semi structured questionnaire and observational checklists were used for collection of data as a part of outbreak investigations (Annex XVI). A draft questionnaire was developed to collect information about the geographic location, farm type, animal species affected, history of the disease and community practices during anthrax outbreak.

3.4. Biosafety Procedure

Standard biosafety procedure like use of proper personal protective equipment's (PPE) before specimen collections, during staining, culture and DNA extractions were used. In addition to laboratory coat/gown, double gloving, and a respiratory cover (such as N95 respirators) with disposable covers for feet were used. Double gloving was used as the outer glove can be changed easily without exposing the skin to any hazardous material. This also helps prevent cross contamination of other animal samples, if sampling is necessary for more than one carcass. A respiratory protection such as N95 respirators was used during culture, staining and for old specimens such as hides or bones that have the potential to create dust as recommended by WHO (2008).

Culture, staining and inactivation of bacterial isolates were performed in biosafety level 3 (BSL3) laboratory and swab extraction and real-time PCR procedures were conducted in

class II, type A2 biological safety cabinets and additional precautions were included the use of powered air-purifying respirators and protective laboratory clothing. All removed PPE was disposed in biohazard waste then autoclaved and all work surfaces and equipment's were decontaminated with fresh 10% bleach for 15 minutes (Annex XII).

3.5. Sample Collection and Transportation

The approach to take samples from animal depends on the type of specimen being examined. In this study, a total of 63 samples were collected from fresh, contaminated dry meat, old and decomposed animal carcasses/product, and from environmental samples as described by WHO (2008).

Of the 63 samples, 24 tissues, 33 swabs, 2 slides, 2 water and 2 bloody soil samples and all were collected aseptically. From putrified carcass, piece of highly vascularized tissue (swabs of nasal turbinates and eye socket) were collected. Swabs of nasal turbinates, eye socket, and soil from where body fluid is believed to have fallen were collected together with very old carcass, hides, bones, and soil under carcass.

Sampling date, sample type, location of sampling point and animal identification were labelled and packed with triple packaging system. Tissue samples (ear and eye lid clipping) were transported under cold chain. Swabs of nasal turbinates, eye socket, fresh bloody materials, visible bloody soil and smear made were transported at room temperature.

3.6. Bacterial isolation and Identifications

3.6.1. Gram-stain

Gram's staining was performed from nasal and rectal swabs to determine the size, shape and arrangements of *B. anthracis*. Gram reaction of the isolated bacteria was conducted according to Merchant & Packer (1967) and Bergey *et al.* (1994). *B. anthracis* was checked for Gram-positive, long, straight bacilli with square and truncated ends with parallel sides found usually single, in pairs or chains of 3 or 4 bacilli (OIE, 2018).

3.6.2. Cultural Examination

A total of 61 samples were cultured on 5% Sheep Blood Agar for isolation of *B. anthracis* bacteria. Swab samples were directly cultured on Blood Agar and 1g of tissue (contaminated dry meat) samples were cut into small pieces and mixed with 10ml phosphate buffered saline (PBS). The suspension was centrifuged and the pellet was spread on 5% sheep blood agar (BA) at 35-37°c for 18-24 hours (WHO, 2008).

One gram of bloody soil was suspended in 10 ml sterile distilled water and suspensions were shaken at 4 °C for 1 hours then filtered through a plug of gauze. Filtered 1 ml suspension were suspended in to 9 ml sterile distilled water and placed in water bath at 65 °C for 15 minutes to kill all vegetative forms. A loopful diluted suspension was cultured on predried Blood Agar plates and incubated at 37°c for 24 hours (WHO, 2008).

After incubation, growth on Sheep Blood Agar was characterized for colony morphology (grey/white, flat colonies, 2-5 mm in diameter, with irregular edges). The hemolytic pattern of the bacteria was categorized according to the types of hemolysis produced on blood agar (Carter, 1986). Non hemolytic colonies were further characterized by Gram staining, spore staining, motility, penicillin susceptibility, capsule production and real-time PCR (Mathias, 2009).

3.6.3. Inducing Capsule Production and Giemsa stain

Single pure colonies were cultured on Nutrient Agar supplemented with 0.7% sodium bicarbonate to detect capsule production (Annex VII). The agar was incubated overnight at 37ºC under a candle jar (to generate 5% CO_2). Mucoid and smooth colonies were characterized and variant was correlated with capsule production ability of *B. anthracis* on 0.7% Sodium bicarbonate nutrient agar (WHO, 2008).

Giemsa stain is a simple stain used to demonstrate the capsule of *B. anthracis*. Thin smears were prepared from cultured isolates on Nutrient Agar supplemented with 0.7% sodium bicarbonate and smeared on a microscope slide and allowed to dry and then fixed by absolute methanol alcohol for 3 minutes. Giemsa was applied to the smear for 60 minutes, wash off stain with water using wash bottle (into hypochlorite solution) and allowed to dry. The slides were viewed at 100x with oil immersion (Quinn *et al*., 1994). After staining, a bacilli was appeared blue, square-ended rods in short chains surrounded by a pinkish red capsule.

3.6.4. Modified Ziehl-Neelsen stain for spores

Smears were made from cultured blood agar, air-dry and fixed with heat and covered the smear with carbol fuchsin based on WHO (2008). Heat the slide for 3-5 minutes; the stain was not boiled and washed off the stain with water using wash bottle (into hypochlorite solution). The slides were decolorized with alcohol until all traces of red are removed and washed off stain with water using wash bottle (into hypochlorite solution). Counter stained with methylene blue for 2 minutes and washed again (into hypochlorite solution) and allowed for drying. The spores were observed using professional trinocular compound microscope with camera fitting (Euromex, iscope) under oil immersion at 100X magnification. Spores stained red and vegetative forms stained blue.

3.6.5. Malachite stain for spores

Smears were made from colonies cultured on blood agar, air-dried and fixed with heat and the slide was placed in a moist chamber (filter paper moistened in a petri dish), covered the smear with 5% aqueous solution of malachite green and left for 60 minutes to act. Stain was washed off with water using wash bottle (into hypochlorite solution) and counter stained with diluted carbol fuchsin for 30 seconds, washed again (into hypochlorite solution) and allowed to dry (WHO, 2008). The spores were observed under oil immersion and spores stained green and the vegetative bacilli stained red.

3.6.6. Biochemical Tests

Motility was observed by motility test medium with triphenyltetrazolium chloride and *B. anthracis* suspected culture isolates were stab inoculated using a straight wire down the middle of motility medium (Semi-solid) for motility test. Organisms migrated from the stab line and diffused into the medium, causing turbidity with formation of red color was considered as positive for motility and organisms grown only along stab line with surrounding medium remaining relatively clear was negative for motility test (Quinn *et al*., 1994). *E. coli* (ATCC 25922) and uninoculated were used as positive and negative control, respectively.

3.6.7. Susceptibility to penicillin G

Sensitivity test of isolated *B. anthracis* colonies were performed on Muller-hinton agar (Annex IX) using disc diffusion method (OIE, 2012). Zone interpretations for *B. anthracis* have not been established by Clinical and Laboratory Standards Institute (CLSI). Hence we used the interpretative diameter zone for non-fastidious microorganisms (cephalosporins) and for staphylococci (penicillin). Depending on the zone of inhibition results were interpreted as susceptible, intermediary susceptible and resistant as recommended by CLSI (M100 S-17) (2018). *B. anthracis* sensitivity to

penicillin G10 IU was conducted on Muller-Hinton agar. Commercially available penicillin G discs of 10 IU (Oxoid, UK) were used to observe the sensitivity.

3.7. Molecular Detections

From a total of 63 samples collected, 61 cultured isolates and 57 directly extracted DNA samples were used in real-time PCR for molecular detection of *pag* and *capC* genes. All cultured isolates were checked for Gram-positive and rods shaped. These samples included, swabs (fresh and old carcasses), tissues (including dried meat) and environmental samples (water and bloody soils).

3.1.1. DNA preparation from bacterial isolates

A single bacterial colony was taken from blood agar plates with 1µl bacterial inoculating loop and mixed with 200 µl 10mM Tris-HCL buffer (tissue preparation kit, Qiagen, Germany). Suspensions culture was heated at 95°C for 20 minutes in water bath and the lysed was transferred to a sterile 1.5ml micro centrifuge tube with 0.22-µm centrifugal filter units (Millipore, Bedford) and centrifuged at 8000rpm for 2 minutes, and stayed for 1 minute to filter the spore and the filter was discarded (Hoffmaster *et al*., 2002). DNA was used immediately for PCR or stored at -20°C. DNA obtained from cell lysed was tested with Real-Time PCR as described by Ellerbrok *et al*. (2002).

3.7.1. Procedure for DNA extraction from swab and tissue

DNA purifications were performed with the QIAamp DNA blood mini kit (Qiagen, Germany), which utilizes silica spin-filter technology for DNA purification. The procedures were carried out according to the manufacturer's instructions (Dauphin and Bowen, 2009). DNA was extracted from all swab and tissue samples collected during the outbreaks according to the protocol described in (Annex IV). The isolated DNA samples were stored at -20°C until real-time PCR was performed.

3.7.2. Real-Time PCR

All real time PCRs was performed in duplicate for capsule and protective antigen detection. The real-time PCR reaction mix (Ellerbrok *et al*., 2002) of capsule consisted of 12.5 µl of Universal Master Mix (Perkin Elmer) containing dNUTPs, MgCl2, reaction buffer and Ampli Taq Gold, 1 µl of 300 nM primer of Cap-forward (5'ACGTATGGTGTTTCAAGATTCATG-3'), 1µl of 300 nM of Cap-Reverse (5' ATTTTCGTCTCATTCTACCTCACC-3'), 1µl of 100 nM of Cap- probe 5'-CCACGGAATTCAAAAATCTCAAATGGCAT- 3'(BHQ 1), 2.5 µl of 1x of 10x Exo Internal Positive control (IPC) Mix, 0.5 µl of 1x of 50x EXO IPC DNA and 1.5 of water.

The real-time PCR reaction mix of protective antigen was consisted of 12.5 µl of Universal Master Mix (Perkin Elmer) containing dNUTPs, MgCl2, reaction buffer and Ampli Taq Gold, 1µl of 300 nM primer of PA-forward (5'CGGATCAAGTATATGGGAATATAGCAA-3'), 1 µl of 300 nM of PA-Reverse (5'CCGGTTTAGTCGTTTCTAATGGAT-3'), 1 µl of 100 nM of probe-PA (5'-CTCGAACTGGAGTGAAGTGTTACCGCAAAT-3'(BHQ1), 2.5 µl of 1x of 10x Exo IPC Mix, 0.5 µl of 1x of 50x EXO IPC DNA and 1.5 of water. A total volume of 20 µl of each master mix added into pre-designated wells of a Micro AMP 96-well microtiter plate.

5µl extracted DNA, 5 µl positive controls and 5µl nucleus free water as no template control (NTC) were added. Positive control of pLepBaBP + plasmid DNA (Sample of known DNA that contains the target of interest instead of virulent strain and allow a positive control without culture). The plates were sealed tightly by applying MicroAmp™ optical adhesive film using the film applicator, thus avoiding potential contamination with anthrax spores.

Real-time PCRs was performed using the 7500 fast real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, and Singapore). For the TaqMan assay, the Standard 7500 operational setting was used with a thermo cycling profile consisting of a

hot-start Taq activation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds (s) and 60°C for 60s (data collection). For the real-time PCR assays, data collection and analysis was performed using the 7500 Fast system sequence detection software and PCRs was performed in a 25 ml final volume. Real-time PCR results were interpreted as positive when fluorescence signal of *Pag*, *Cap* and all IPC were exhibited growth curves within 40 cycles and negative when fluorescence signals of *Pag*, *Cap* and NTC were did not exhibit within 40 cycle (Ellerbrok *et al*., 2002).

3.8. Data Analysis

Data describing anthrax outbreak in sex, age, species and season was classified, filtered, coded and entered in to Microsoft Excel® 2007. Frequency table was produced to present the study findings.

4. RESULTS

4.1. Series of case and epidemiological scenario

The *first* outbreak occurred in Illubabor zone, Darimu district, Enariyo kebele, in March, 2019. The first cases appeared toward the beginning of March, 2019 and 16 cattle's, 4 goats and 2 donkeys were died and no human cases were reported.

The *second* outbreak occurred in south Gonder, Farta District, Wowa Kebele in April, 2019. The most recent previous outbreak reported in area occurred in 2018, but not in the same district. The first cases appeared toward the beginning of April 2019. The outbreak began after a period of heavy rainfall that followed the long dry season of the country. Death of one goat and one human cases of anthrax was occurred.

Third outbreak was occurred in Wolayita Zone, Damota-Sore District, Gurmuledisa Kebele in beginning of April 2019. The livestock of one farm in the Kebele was affected. One cow died with suddenly, oozing of blood through natural orifices and bloating and no human cases were occurred. The cattle in this area were vaccinated. The fourth outbreaks occurred in Gurage zone, Abeshege District, Ghibe Sheleko National Park in wild animals (i.e. hippopotamus) in at the end of April, 2019. A total of 28 hippopotamus died but no human cases were reported (Annex XV).

The *fifth* anthrax suspected outbreak was investigated in two districts of Zone 4 of the Afar Region in Ewa and Awra district in three kebele at the end of May, 2019. A mass death of more than 600 sheep and goats were reported on May 22, 2019 following a prolonged hot dry spell, which in turn was preceded by heavy rain falls and flooding in the area.

4.2. Cultural examination

In this study, a total of 61 (24 tissues, 33 swabs, 2 bloody soils and 2 water) samples were cultured on 5% Sheep Blood Agar for isolation of *B. anthracis* and a total of five outbreaks were investigated. From cultured samples, 9.8% (6/61) dried meat (*Quwanta*) samples were positive for culture from goat died from anthrax suspected samples (Table 5).

The bacterium was grown readily on blood agar as pure culture. On Blood Agar, the colonies were non haemolytic, flat, dry, grayish and tenacious colonies (Figure 10). The colonies of *B. anthracis* isolate was distinct from other *Bacillus* isolates as they were characterized by a "medusa head", which appeared as curl-like projections.

Table 5: Number of samples collected and cultured positive samples from different anthrax suspected outbreak investigations in animals.

Region	Zone	District	Kebele	Animal species affected	No. animal died	Type of sample cultured	Number of samples cultured	Culture positive samples	Culture Positivity %
Oromia	Illubabor	Darimu	Enariyo	Bovine	16	Swabs from eye socket and nasal turbinate	10	0	0
				Goat	4				
				Donkey	2				
Amhara	South Gonder	Farta	Wowa	Caprine	1	Dried meat (Quwanta)	6	6	100
SNNPR	Wolayita sodo	Damota sore	Gurmuledisa	Bovine	1	Tissue	4	0	0
						Nasal and rectal swab	4	0	0
	Gurage	Abeshege	Ghibe Yemengisti Ersha	Hippopotamus	28	Swab	10	0	0
						Tissue	4		0
						Water	2	0	0
Afar	4	Ewa	Sunuta & Dulo	Ovine & Caprine	450	Swab, tissue & bloody soil	19	0	0
		Awura	Awura	Ovine & Caprine	150	Swab and bloody soil	2	0	0
Total					**652**		**61**	**6**	9.8%

No = number, % = Percent

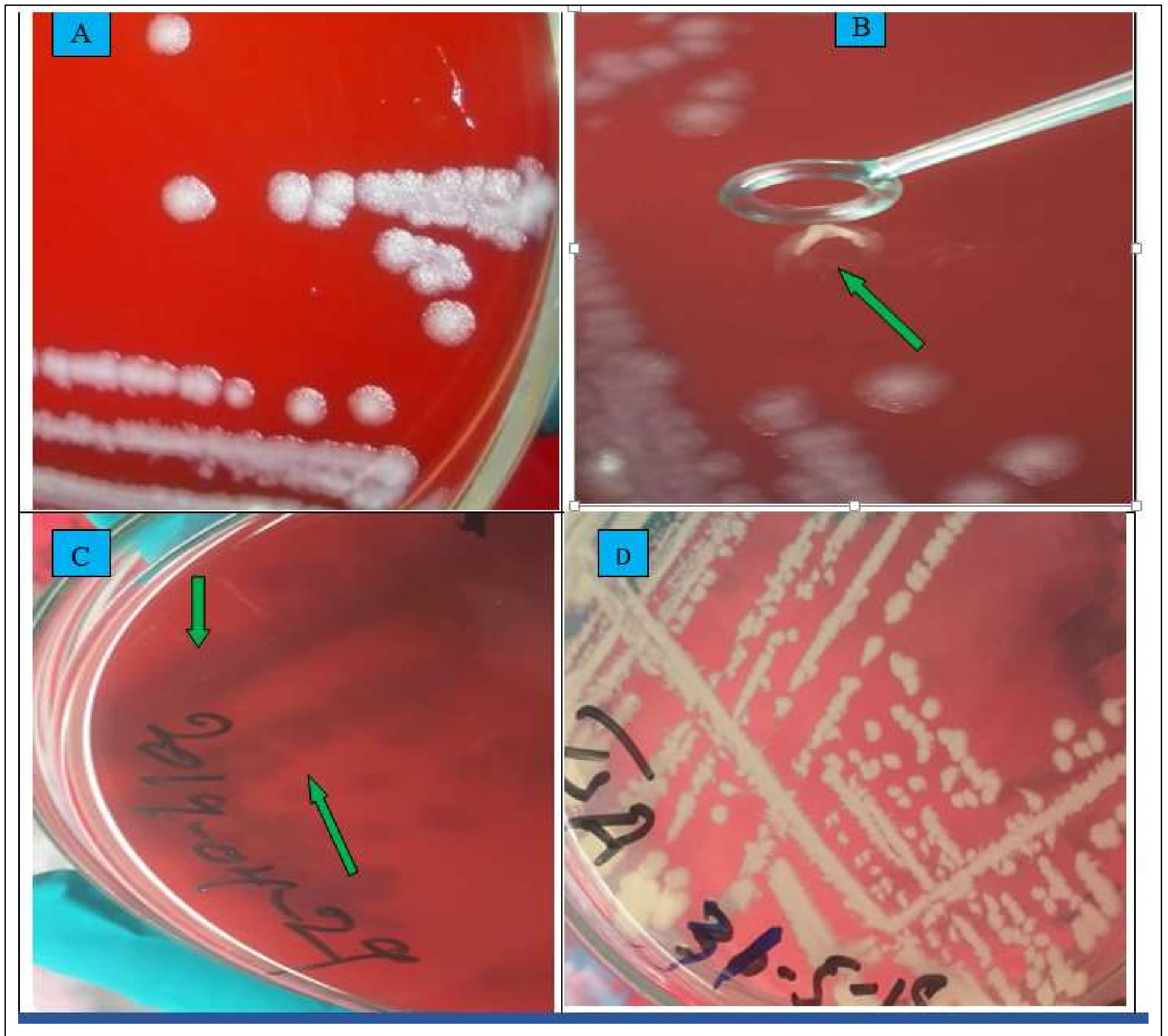

Figure 10**:** Colony morphology of *B. anthracis* on Sheep Blood agar. (A) With gray, flat, dry irregular edges colonies. (B)The arrow show tenacious colonies (C) non-haemolytic appearance colonies and (D) growth of *B. anthracis* on tryptic soya agar.

4.3. Gram-staining

Gram-positive, rod bacilli and long chains of *B. anthracis* was not observed from two swab samples (nasal swab and rectal) prepared in the field and stained by Gram staining.

Based on colony morphology and suspected colonies, six cultured isolates were observed with gram staining and were typically Gram-positive, thick, long, straight bacilli with square or truncated ends with parallel sides found usually single, in pairs or long chains of bacilli in appearance (Figure 11) and spores were appeared as unstained areas within the cell located centrally, subterminal and ellipsoidal.

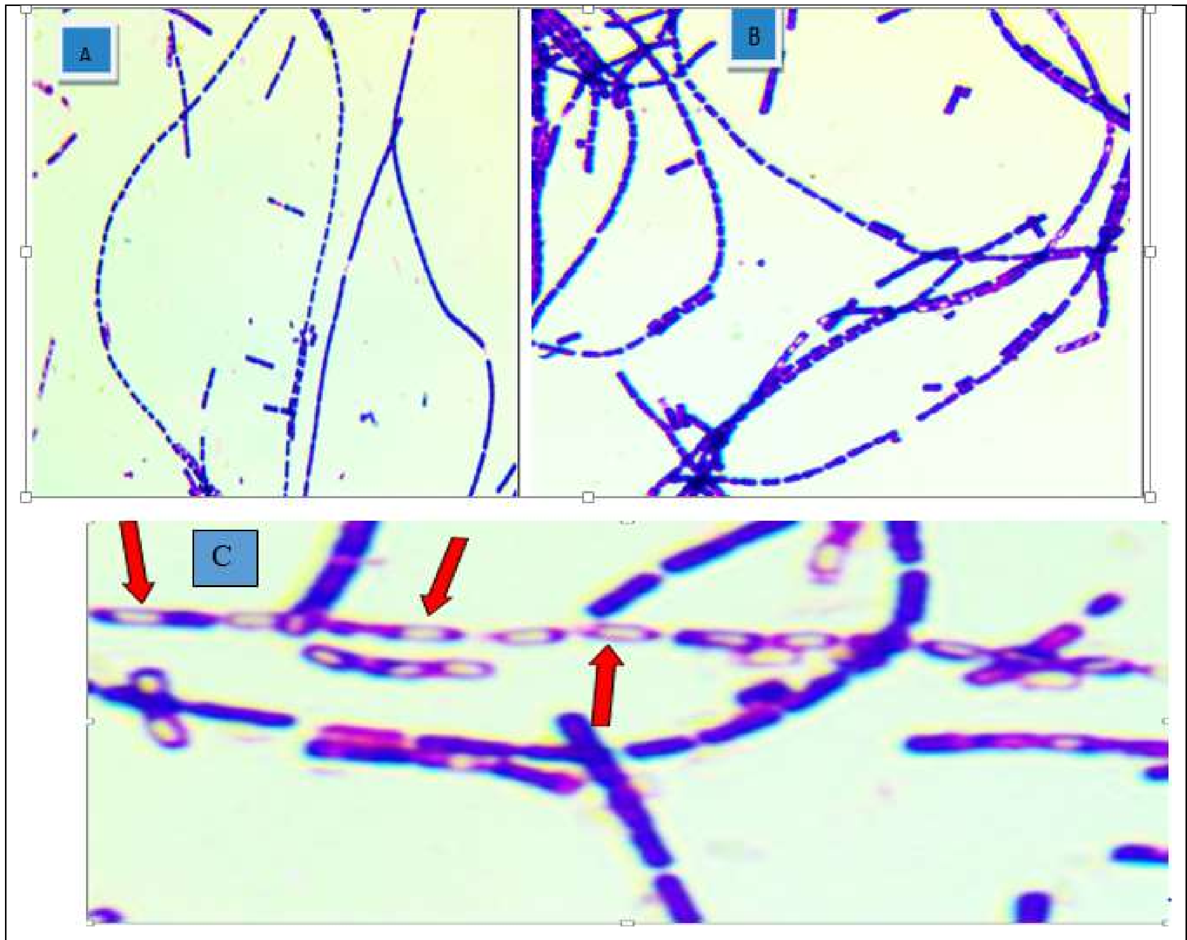

Figure 11**:** Gram-staining results of *B. anthracis* colonies from cultured Blood Agar: (A and B) show Gram-positive rods in long chains. (C) Show vegetative bacilli and spores (the arrow) appeared as unstained and centrally located.

4.4. Motility Test

B. anthracis suspected cultured isolates were grown only along stab line (Figure 12) hence, were found to be non-motile. Motile *E. coli* was used as positive control and spread throughout the medium as expected.

Figure 12: Motility test: (A) *E. coli* (positive control) was motile, (B) *B. anthracis* isolate was non-motile (Arrow shows stab line), (C) un-inoculated negative control.

4.5. Penicillin susceptibility

The zone of inhibition around the disc was measured (Figure 13). Susceptibility to penicillin was indicated by a zone of inhibition of growth (33.68 mm) around the disc and cultured positive isolates were susceptible for penicillin G.

Figure 13: Susceptibility test (Zone of inhibition of *B. anthracis* isolate) by penicillin G

4.6. Modified Ziehl-Neelsen Stain for Spores

Culture positive isolates were positive for spore formation (Figure 13). The spores of *B. anthracis* belonged to *Bacillus* species morphological group 1, characterized by spores being centrally or sub terminally positioned.

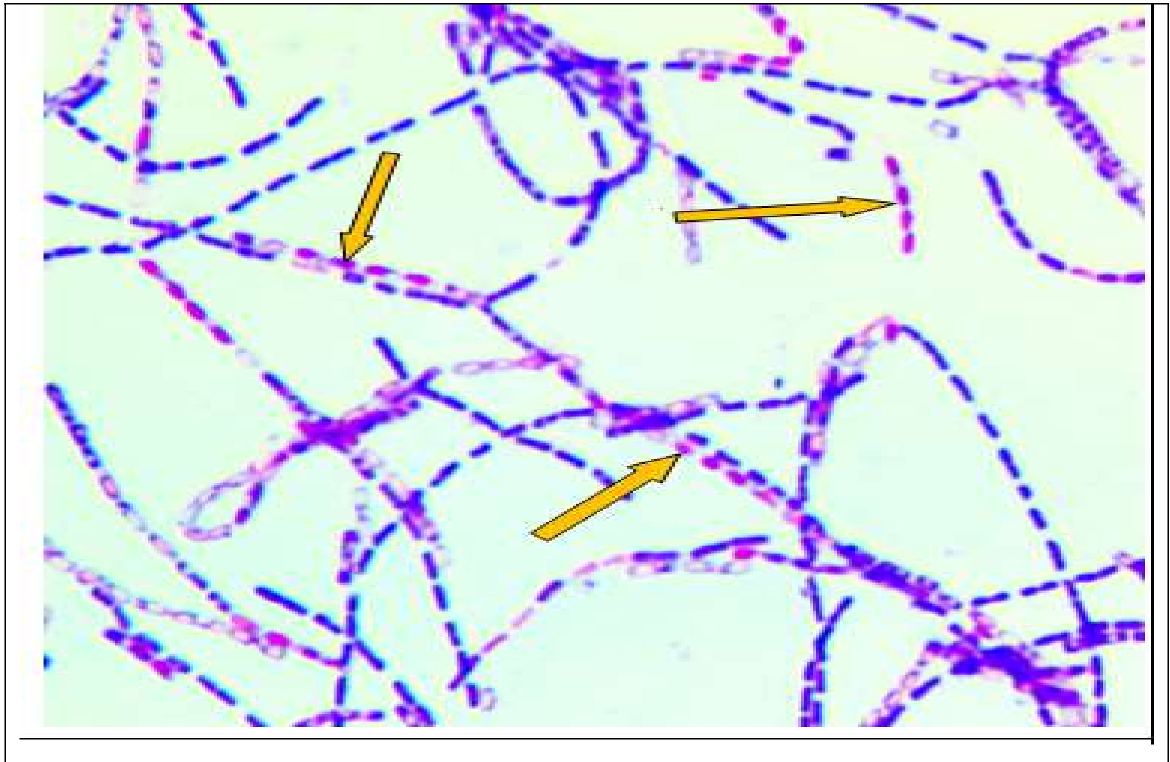

Figure 14: Modified Ziehl-Neelsen stain of *B. anthracis* demonstrating spores. Spores (arrow) stained red and vegetative bacilli stained blue.

4.7. Malachite Green Stain for Spores

Form this study, cultured positive isolates (*B. anthracis*) were subjected to Malachite green stain to demonstrate the spore and the spore stained green and the vegetative forms stained red (Figure, 15).

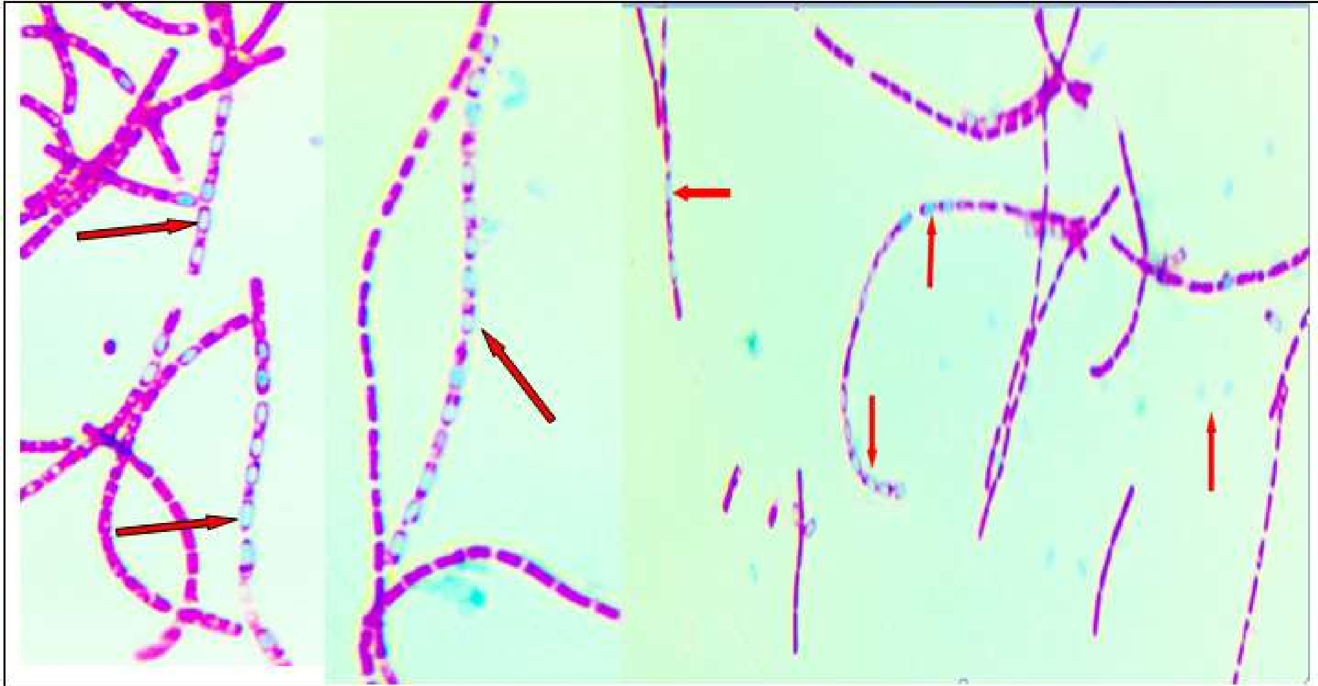

Figure 15: Malachite green stain: The spore (arrow) stained green and the vegetative from stained pink.

4.8. Capsule Production and Capsule Staining

Capsule was induced by growth in nutrient agar containing 0.7% sodium bicarbonate and incubated at 37^0C under candle jar for 24 hours. Mucoid colonies were observed and Giemsa staining were prepared from bicarbonate agar. The bacilli was stained that appeared blue, square-ended rods in short chains surrounded by a pinkish red capsule (Figure 16).

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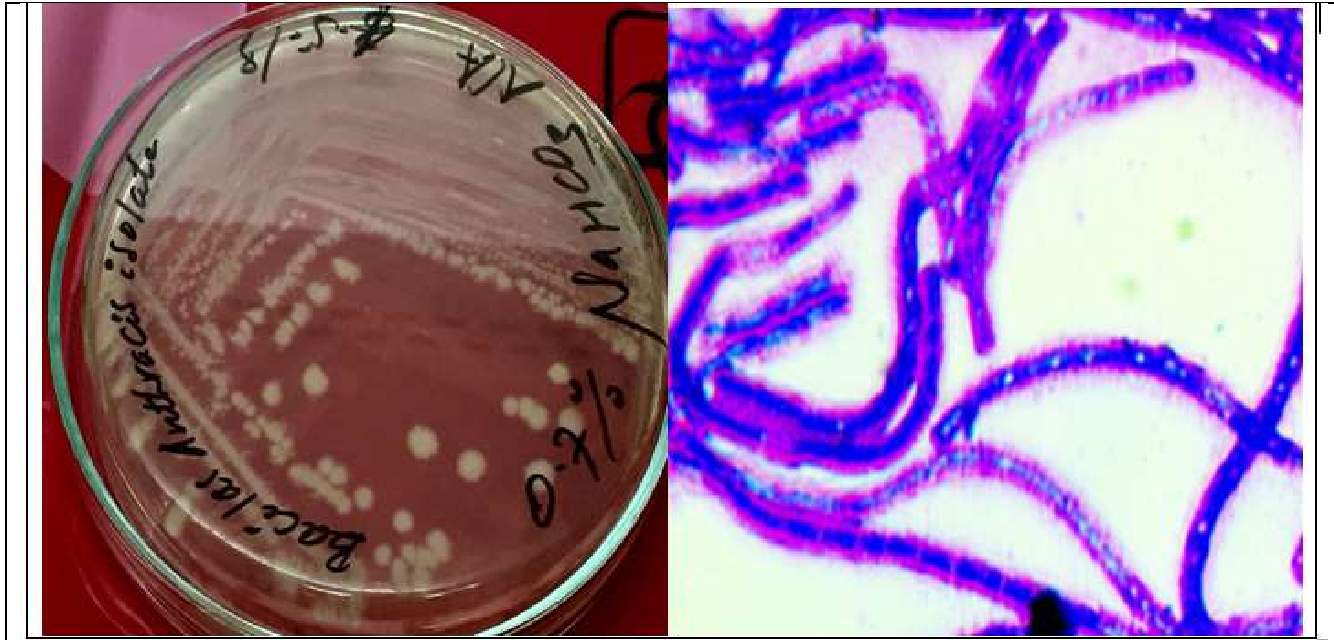

Figure 16: Colony morphology of *B. anthracis* on Nutrient Agar containing 0.7% sodium bicarbonate and Geimsa staining; (Left) Mucoid colonies and (Right) capsule stained pinkish red and bacilli stained blue with Giemsa stain.

4.9. Real-Time PCR

From a total of 61 cultured isolate, 9.8% (6/61) were positive for real-time PCR and all directly extracted DNA's from samples were negative by real-time PCR. The dried meat samples (Quwanta) were first cultured on Blood Agar and DNA was extracted from the bacterial isolates and then detected with PCR. Both virulent plasmid markers *pag* (on plasmid pXO1) and *cap-C* (on plasmid pXO2) were detected from suspicious colonies of *B. anthracis* isolates. In all cases, fluorescence signals of the *pag* marker appeared early, with cycle threshold (*CT*) values of 19.63 to 21.35 (Figure 16), whereas signals of the internal IPC were delayed with *CT* values of 27.58 to 28.44. The *cap-C* gene marker was positive in PCR assays with *CT* values of 30.93 to 33.18 (Annex VI).

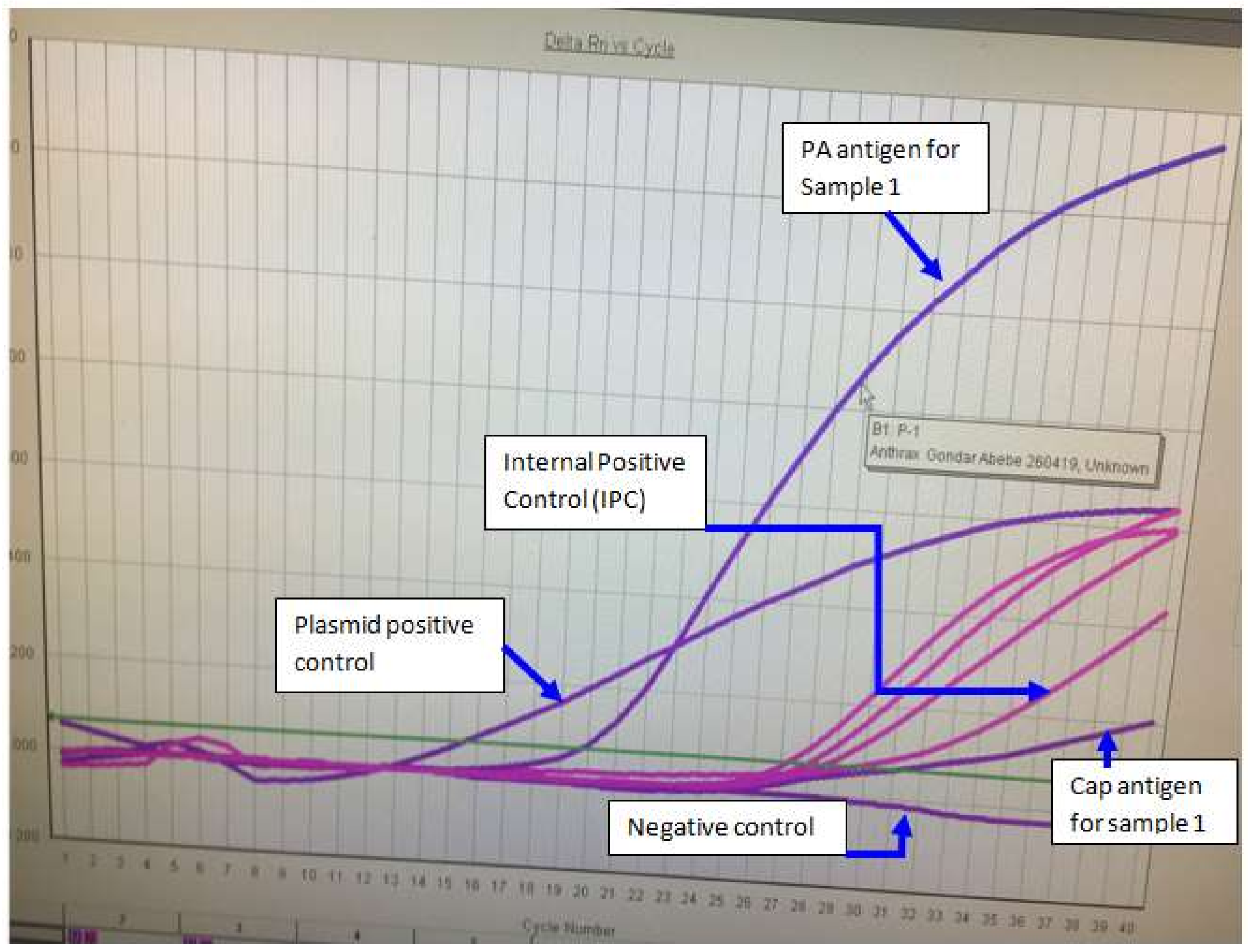

Figure 17: Real-time PCR Result shows *Pag*, *cap*-C and IPC standards were fluorescence signals exhibited growth curves within 40 cycles.

5. DISCUSSION

This study aimed at identification of anthrax outbreak hotspot areas through laboratory confirmation from different parts of the country. Hence, a total of 63 samples were collected from five reported outbreaks and only 9.8% (6/63) samples collected from South Gonder, Farta District were confirmed by laboratory to be caused by *B. anthracis.* The laboratory confirmation of *B. anthracis*es the causal agent of anthrax were carried out by using Gram-staining, culture, capsule and spore staining, biochemical tests and molecular detection of targeted virulent genes by real-time PCR. Similarly, in the country Shiferaw (2004) has also cultured and isolated *B. anthracis* from case of sudden death in goat in Wabessa village, Dessie Zuria. Based on the report of Shiferaw (2004), the samples were cultured and colony morphology was characterized, Gram-staining, biochemical test and pathogenesis tests were conducted to confirm the causal agent of anthrax.

During the study period, five anthrax suspected outbreaks were reported by the passive surveillance system, however from this only one of the outbreaks were confirmed by laboratory test to be caused by *B. anthracis* and the rest were negative. In the country previous retrospective record review from 2009-2013 showed that within five years a total of 26737 animal cases with 8523 animal deaths due to anthrax were reported (Bahiru *et al*., 2016). These data showed that each year on average death of 1705 animals were recorded due to anthrax. The data was compiled from passive surveillance reports and those reports were based on history and clinical signs and many of suspected cases of livestock anthrax are reported from several districts without confirmation by laboratories and any sudden death in herbivorous animals is reported as anthrax suspected outbreaks.

WHO (2008) reported that all causes of sudden death in animals such as clostridial diseases, peracute pasteurellosis, nutritional deficiency or poisoning, sudden widespread deaths during environmentally stressful conditions such as lightning strike or electrocution, sudden severe drops in environmental temperature and severe droughts, especially when it occurs in a group context, may be confused with the peracute and

acute forms of anthrax. So the use of proper case definition for anthrax in livestock and laboratory confirmation of suspected cases are important to know the real magnitude and impact of anthrax in the country.

The disease being characterized by sudden death of animals and the chance of getting fresh samples is questionable and nearby veterinary clinics have higher chance of accessing the proper samples on time. Thus accessing samples timely was challenge of this study.

In the present study, cultured *B. anthracis* isolate was found gray/white, non-hemolytic colonies on blood agar and similar findings were reported by Shiferaw (2004) in Dessie Zuria, Wabessa Village, Lekota *et al*. (2016) report in Northern Cape Province and Kruger National Park of South Africa. In addition, the spore forming nature of the isolate was confirmed by spore staining using different staining which was located centrally. *B. anthracis* forms spores upon nutrient starvation, exposed to oxygen and vegetative cells can develop into the dormant spore form of the bacterium. Prolonged culture of *B. anthracis* in a variety of media can also facilitate spore formation (Schaeffer *et al*., 1965; Leighton and Doi, 1971; Turnbull, 2002; Turnbull *et al*., 2007; WHO, 2008).

The non-motility and susceptibility to penicillin G were observed and similar findings were reported by Shiferaw (2004), Marston et al. (2006) and Lekota *et al*. (2018) in the enzootic region of Pafuri, Kruger National Park in the Limpopo Province of South Africa. Motile strains and strains that appear to produce flagella have also been reported (Klee *et al*., 2006; Liang and Yu, 1999) although the genomes of sequenced strains, which are none motile and presumed non flagellated, are devoid of some genes commonly associated with flagella (Read *et al*., 2003). Rare penicillin-resistant isolates have also been reported (Cavallo *et al*., 2002; Mohammed *et al*., 2002; Klee *et al*., 2006). Sequenced *B. anthracis* strains, which are penicillin- susceptible, harbor two structural genes for b-lactamase (*bla*) proteins (Chen *et al*., 2003; Read *et al*., 2003). These *bla* genes are transcriptionally silent in prototypical strains, but constitutively expressed in a rare highly penicillin-resistant isolate (Chen *et al*., 2004).

The formation of capsule in the *B. anthracis* isolates was induced by growing in Nutrient Agar containing 0.7% sodium bicarbonate with mucoid colonies. This result is in agreement with the previous reports (Green *et al*., 1985; Koehler *et al*., 1994; Sirard *et al*., 1994; Drysdale *et al*., 2004; WHO 2008). Upon Giemsa staining of the prepared smears showed the capsule is reddish with blue bacilli, which is similar to the findings of Quinn *et al*. (1994) and Lekota *et al*. (2018).

The detection of *capC* and *pag* genes in the current study indicates that the isolates were pathogenic as they were isolated from outbreak cases that killed animals. The molecular detections of *B. anthracis* virulent genes by real-time PCR from animal anthrax suspected outbreak was carried out and reported for the first time in the country. Similar findings of such virulent gene detections were reported by Klee *et al*. (2006) from wild great apes in Cote d'Ivoire and Cameroon and who also recommend the use of PCR to confirm *B. anthracis.* The pathogenic characteristics of *B. anthracis* depends on two virulence determinants; the poly-D-glutamic acid capsule and tripartite protein toxin that are encoded for by capsule (*CapA, CapB, CapC)* and toxin *(* pagA, *lef* and cya) genes (Hadjinicolaou *&* Koehle*r,* 2009) on plasmids PXO2 and PXO1, respectively both of which were confirmed in the present study.

Currently, Ethiopia has developed national strategic plan aimed to significantly reduce and ultimately control the public health impact of anthrax in humans and animals in the country through sustained surveillance, laboratory diagnosis, prevention and control systems and community awareness. The vision is to ensure anthrax is no longer a significant public and animal health problem in Ethiopia by 2030. The guiding principle was that Prevention and control of anthrax in animals effectively reduces its impact on public health and the national economy. Hence, in order to achieve this national plan improving the national and regional animal and public health laboratory capacity for anthrax diagnostics is very important. Moreover identification of anthrax outbreak hotspot areas is among the initially planned activities. Once the anthrax outbreak areas

are confirmed by laboratory diagnosis disease prevention and controlling measures like mass vaccination of livestock can be applied for continuous years.

6. CONCULUSION AND RECOMMENDATIONS

In this study, the confirmation of *B. anthracis* from outbreaks indicates that anthrax still remains endemic in the country and causes diseases in livestock. This is the first report in Ethiopia documenting molecular detection of *B. anthracis* targeted virulence genes located on plasmids pXO1 and pXO2 from anthrax suspected outbreak in animals. *B. anthracis* the cause of anthrax was confirmed only from one outbreak out of five anthrax suspected outbreak reports. This indicates that the disease was miss diagnosed with other diseases of similar clinical signs during passive surveillance.

The main source of human infection is direct or indirect contact with infected or contaminated animal products. The occurrence of human infection correlates with the presence of disease in the animal population. Prevention and control of anthrax in animals effectively reduces its impact on public health and the national economy. A multi sectoral collaborative approach and capacity building coupled with efficient communication of cases are essential for successful prevention and control of the disease in the control.

RECOMMENDATION

As the disease is endemic in Ethiopia and mostly under reported and miss diagnosed with other diseases, the following recommendations are forwarded:

- Laboratory confirmation of anthrax suspected outbreaks should be conducted to differentiate the disease from other diseases with similar clinical signs as this assists to know the magnitude of the disease in the country,
- The regional laboratories and veterinary clinics staffs being near for immediate outbreak response should be trained on biosafety, sample collection and diagnosis of anthrax suspected cases and investigation of any sudden or unexpected death in livestock,

- Joint outbreak investigation, proper animal immunization and public awareness should be created regarding the risk of handling and consumption of animals dying of suspected anthrax cases and other unknown cases,

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8. ANNEXES

Annex I: Clinical Description

Suspicion of anthrax was arising from the observation of clinical symptoms, pathological lesions and epidemiology of the disease. Clinical manifestations vary from species to species, presumably reflecting differences in susceptibility. Sudden death in apparently healthy (domestic and wild) animals which may be accompanied by bloody discharges from natural orifices, rapid bloating of the carcass, incomplete rigor mortis and the absence of clotting of the blood was the characteristics of anthrax in susceptible animals. In more resistant species, local signs such as swellings of the oral and pharyngeal region are seen (WHO, 2008).

Annex II: Criteria for Laboratory Diagnosis of Anthrax

In suspected laboratory diagnosis, the smear shows Gram-positive, square-ended rods in pairs or short chains, occasionally singly, in association with a suggestive clinical history. Presumptive laboratory diagnosis includes smear stained with poly-chrome methylene blue shows dark blue square-ended rods in pairs or short chains, occasionally singly, surrounded by pink capsule. A confirmatory laboratory diagnosis test shows culture of *B. anthracis* and confirms toxin and capsule genes by PCR (WHO, 2008).

Annex III: Procedure for demonstration of *B. anthracis* in stained smears using Gram's stain

Make two thin smears of clinical material from swabs or from a small drop of blood

2. Air dry the smear
3. Fix gently by passing over a flame
4. Cover the smear with Gention violet and leave it for one minute

5. Rinse the slide gently with tap or distilled water into container containing hypochlorite solution
6. Cover the slide with Gram's iodine and leave it for one minute
7. Rinse the slide gently with tap or distilled water into container containing hypochlorite solution
8. Cover the smear with acetone for 30 seconds
9. Rinse the slide gently with tap or distilled water into container containing hypochlorite solution
10. Cover the smear with Safranine and leave it for one minute
11. Rinse the slide gently with tap or distilled water into container containing hypochlorite solution
12. Air dry the slide and examine under 100X objective
13. Examine for any Gram positive *anthrax bacilli*

Annex IV: Procedure for DNA extraction from swabs and tissues samples protocol

1. Wearing all appropriate PPE
2. Spray down the BSC work surface area with the 0.5% hypochlorite solution, wait 10 minutes and wipe away
3. Repeat with 70% ethanol and wipe away
4. Place all reagents and supplies in the BSC
5. Disinfect all instruments before use by spraying with 0.5% hypochlorite solution and repeat with 70% alcohol
6. Specimens were prepared as follows

6.1 Swabs

6.1.1. 400µl PBS was added to a 2.0ml micro centrifuge tube with O-ring

6.1.2. The shaft of the swab was cut approximately 1.0cm above the absorbent materials

6.1.3. Absorbent materials were placed in the micro centrifuge tube containing 400µl PBS

6.1.4. Vortex on high for 2 minutes

6.1.5. Hydrate the swab for 10 minutes and making sure the entire swab is within the PBS

6.1.6. The swabs were transferred by using forceps into SETS collection tube (outer tube with inner tube)

6.1.7. Remaining PBS were transferred into SETS collection tube using a pipet with the swab

6.1.8. SETS collection tube were placed in a microcentrifuge rotory and centrifuged for 1 minute at 10000xg to collect the elute in the outer tube.

6.1.9. The rotory was opened in the BSC and the tubes were removed.

6.1.10. The swab was transferred using sterile forceps back to the micro centrifuge tube for storage.

6.1.11. The inner SETS tube was discarded and fasten screw cap tightly.

6.1.12. 20µl of Qiagen protease was added to the bottom of an empty 1.5mlmicrocenterfuge tube

6.1.13. 200µl of the prepared specimen was added to the tube with protease.

6.1.14. 200µl Buffer AL were added to the samples and Mixed by puls-vortexing for 15 minutes

6.1.15. The tube was placed in the microcenterfuge rotory in BSC, and centrifuged for 30 seconds at 10,000xg

6.1.16. The tubes were removed by opening the rotory

6.1.17. All tubes were decontaminated with 0.5% hypochlorite solution and removed from BSC

6.1.18. The sample tube was incubated at 56ºC for 10 minutes.

6.1.19. The tubes returned to BSC after incubation.

6.2. **Tissue**

6.2.1. The tissue samples were cut up to 25mg into small pieces by using sterile forceps and scissors and placed in a 1.5ml microcenterfuge tube

6.2.2. 180µl Buffer ATL was added and mixed by vortexing

6.2.3. 20µl protinase K was added and mixed by vortexing

6.2.4. The tube was placed in the microcenterfuge rotory in BSC, and centrifuged for 30 seconds at 10,000xg

6.2.5. The rotory was opened in the BSC and the tubes were removed.

6.2.6. All tubes were decontaminated with 0.5% hypochlorite solution and removed from BSC

6.2.7. The sample was incubated at 56ºC until the tissue was completely lysed (for 1-3 hours depending on size)

6.2.8. The sample tube was incubated at 56ºC for 10 minutes.

6.2.9. 200µl Buffer AL were added to the samples and Mixed by puls-vortexing for 15 minutes

6.2.10. The tube was placed in the microcenterfuge rotory in BSC, and centrifuged for 30 seconds at 10,000xg

6.2.11. The tubes were removed by opening the rotory

6.1.17. All tubes were decontaminated with 0.5% hypochlorite solution and removed from BSC

6.1.18. The sample tube was incubated at 70ºC for 10 minutes.

6.1.19. The tubes returned to BSC after incubation

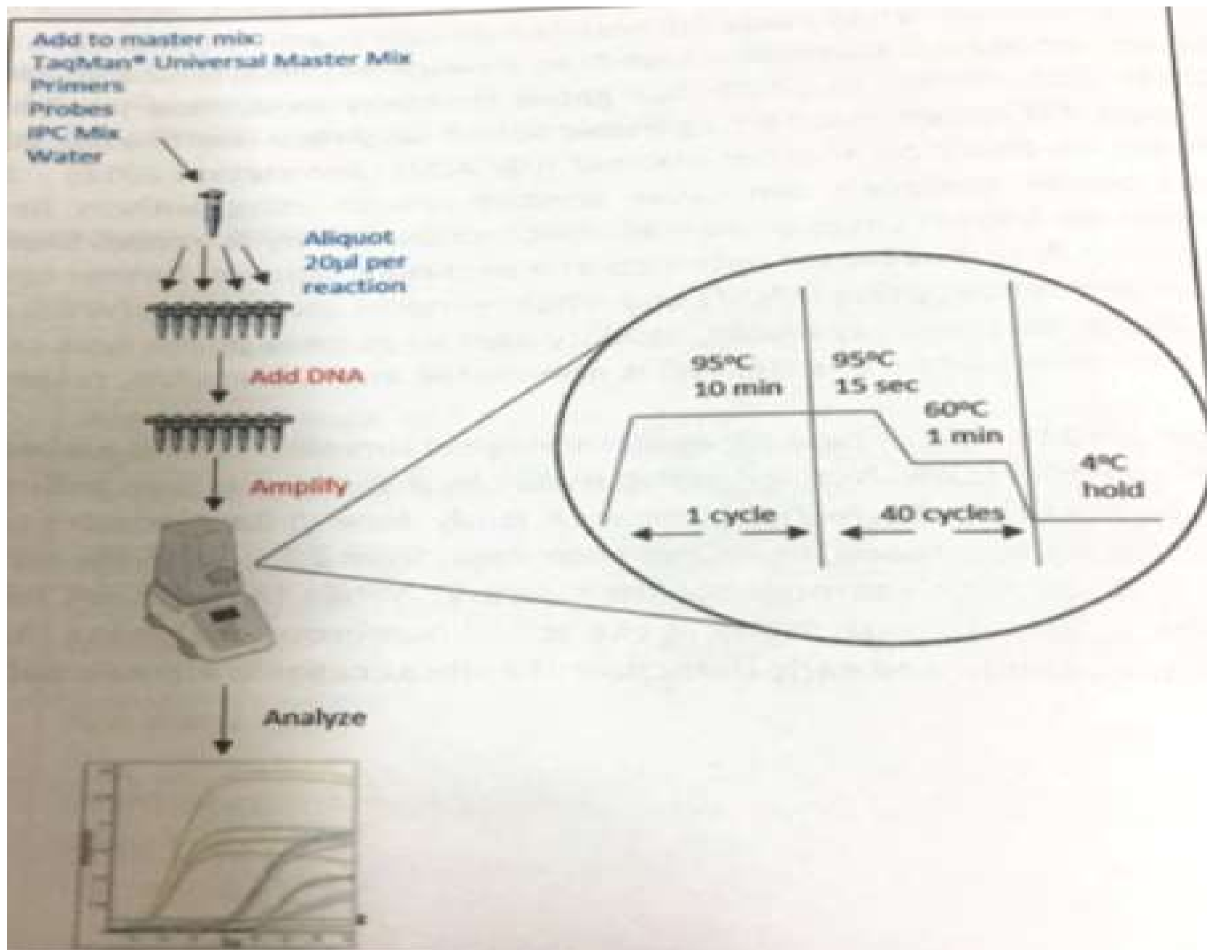
7. Label the appropriate number of Millipore ultrafilter tubes and placed in the BSC
8. The filter tubes were uncapped
9. The lysed swab or tissue were pipette into the filter cup
10. The tube was placed in the microcenterfuge rotory in BSC, and centrifuged for 2 minute at 8,000xg
11. The tubes were removed by opening the rotory
12. The filter was discarded from each tube and gloves were changed
13. The filtered solution was pipette into a new 2.0ml microcenterfuge tube with O-rings for further processing

14. All tubes were decontaminated with 0.5% hypochlorite solution and removed from BSC
15. Repeat wiping by 70% alcohol

Precipitation and Elution of DNA

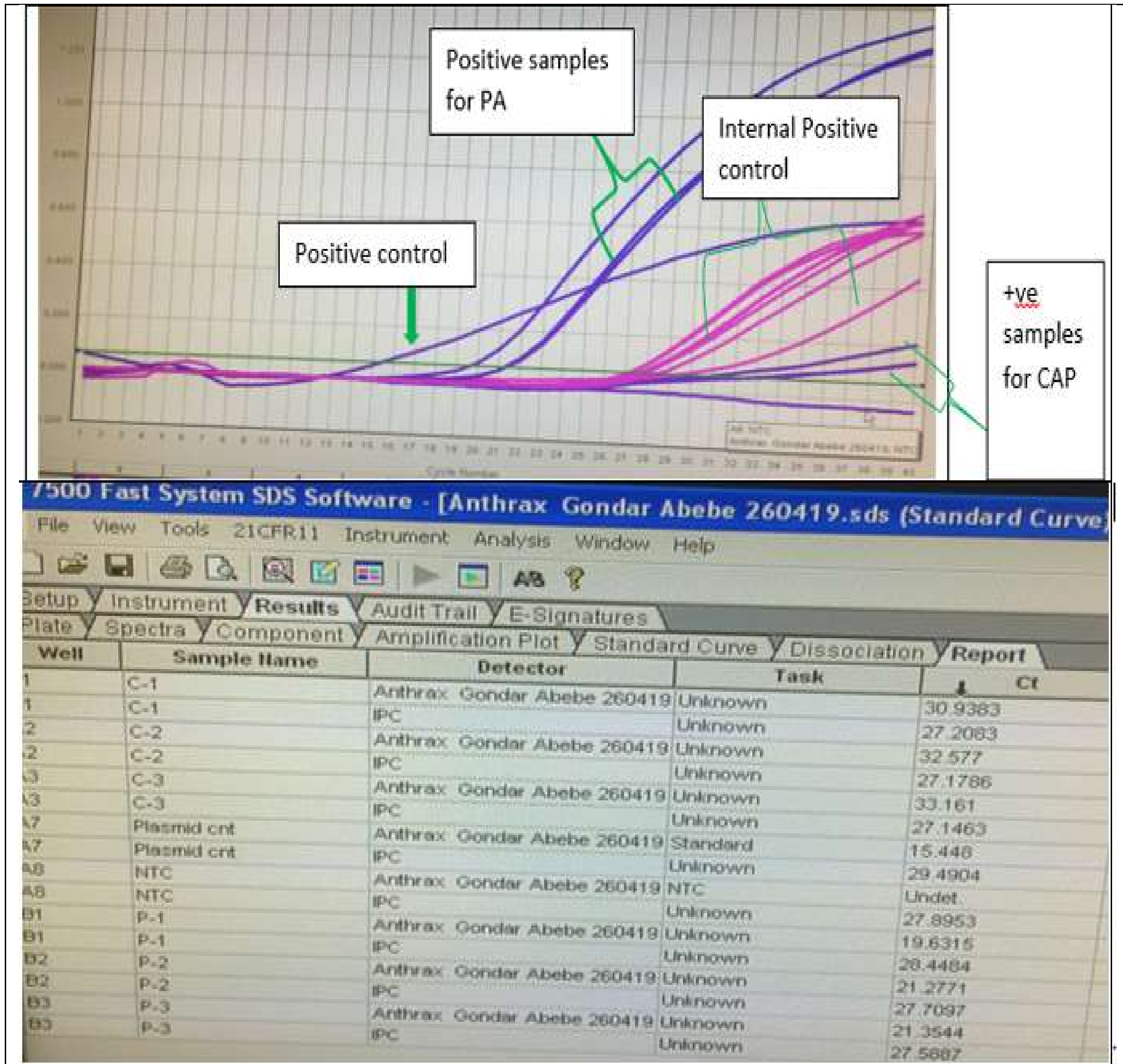
16. 200µl ethanol was added to filtered samples. Pulse-vortex for 15 seconds, followed by a brief centrifuge
17. Contents of the sample tubes were transferred to a QIAamp mini spin column in a2.0ml collection tube without wetting the rim.
18. Centrifuged at 6,000xg for 1 minute.
19. Column was placed in a new 2.0ml collection tube and discarding the old one.
20. The cup was opened and 500µl Buffer AW1 was added to the center of the column filter.
21. Centrifuged at 6,000xg for 1 minute
22. The cup was opened and 500µl Buffer AW2 was added to the center of the column filter.
23. Centrifuged at full 20,000xg for 3 minute
24. Column was placed in a new 1.5ml microcenterfuge tube and discarding the old one
25. 200µl of AE buffer was added to the column and incubated at room temperature for 5 minutes.
26. Centrifuged at 6,000xg for 1 minute
27. The column was discarded and the cap was closed
28. Extracted DNA was stored at -20^{0}C properly until used or processed.

Annex V: Work Flow chart for Real-Time PCR procedure



All steps in blue are completed in a “clean room” while steps in red are completed in a “dirty room” Adapted from Ellerbrok (2002).

Annex VI: Relative fluorescence vs cycle number and cycle threshold (CT) value for Real-Time PCR results



Well	Sample Name	Detector	Task	Ct
1	C-1	Anthrax Gondar Abebe 260419	Unknown	30.9383
1	C-1	IPC	Unknown	27.2083
2	C-2	Anthrax Gondar Abebe 260419	Unknown	32.577
2	C-2	IPC	Unknown	27.1786
A3	C-3	Anthrax Gondar Abebe 260419	Unknown	33.161
A3	C-3	IPC	Unknown	27.1463
A7	Plasmid cnt	Anthrax Gondar Abebe 260419	Standard	15.448
A7	Plasmid cnt	IPC	Unknown	29.4904
A8	NTC	Anthrax Gondar Abebe 260419	NTC	Undet.
A8	NTC	IPC	Unknown	27.8953
B1	P-1	Anthrax Gondar Abebe 260419	Unknown	19.6315
B1	P-1	IPC	Unknown	28.4484
B2	P-2	Anthrax Gondar Abebe 260419	Unknown	21.2771
B2	P-2	IPC	Unknown	27.7097
B3	P-3	Anthrax Gondar Abebe 260419	Unknown	21.3544
B3	P-3	IPC	Unknown	27.5887

Annex VII: Preparation of nutrient agar containing 0.7% sodium bicarbonate

The agar was prepared by weighing nutrient agar base powder required for a final volume of 100 ml but reconstituting the measured agar in only 90 ml of water, then autoclaved and cooled to 50°C in a water bath. 10 ml of a filter-sterilized (0.22µm filter) 7% solution of sodium bicarbonate was added. Mixed and poured into Petri dishes. The encapsulated *B. anthracis* will form mucoid colonies and the capsule can be visualised by making thin

smears on microscope slides, fixing, and staining with polychrome methylene blue (OIE, 2018).

Annex VIII: Giemsa stain preparations

Solution 1: Giemsa stain

Giemsa powder was prepared as follows: 1.0 g of Giemsa powder was dissolved in 66 ml of glycerol at 55-60°C for about 2 hours; 66 ml of methanol was mixed thoroughly.

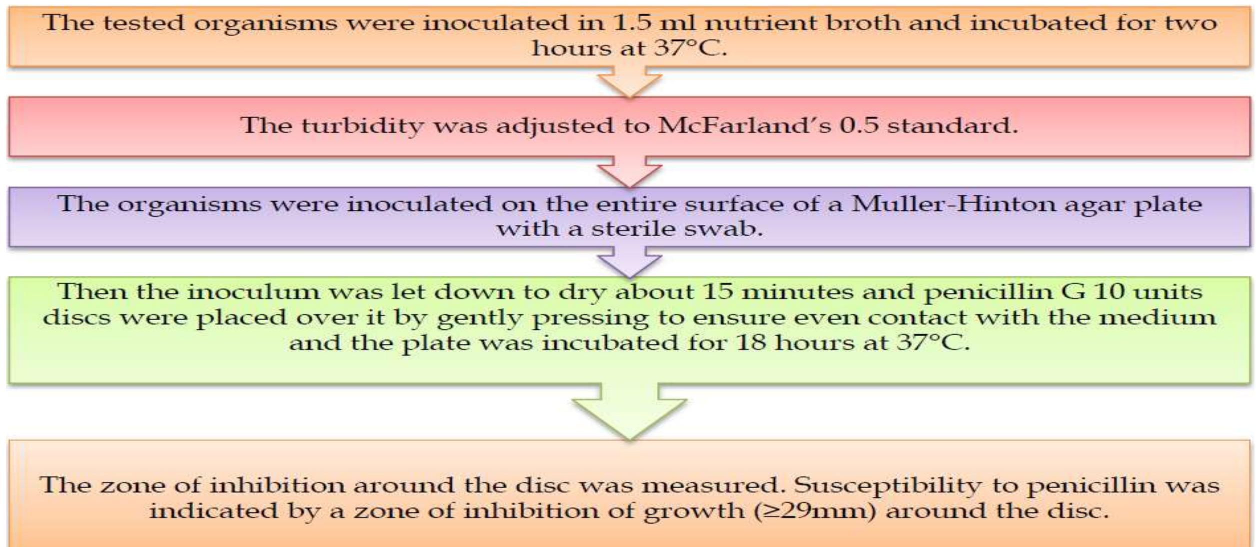
Solution 2: Giemsa buffer

61.1 ml of Sodium phosphate (Na_2HPO_4 (anhydrous M/15 solution (9.47 g/litre or $Na_2HPO_4.2H_2O$, M/15 solution (11.87 g/liter) was mixed with 38.9 ml potassium phosphate, KH_2PO_4 (anhydrous) M/15 solution (9.08 g/liter) with 900 ml distilled water. 1 volume of Giemsa stain solution mixed with 9 volume of buffer solution (Quinn *et al*., 1994).

Annex IX: Phosphate buffered saline solution

For preparation of phosphate buffered saline, 8 grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate (Na2HPO4.12H2O), 0.2 grams of potassium chloride (KCl) and 0.2 gm of potassium hydrogen phosphate (KH2PO4) were suspended in 1000 ml of distilled water, the solution was heated to dissolve completely. The solution was then sterilized by autoclaved and stored for future use.

Annex X: Susceptibility to penicillin G in Müller-Hinton agar



Annex XI: Disinfection, decontamination and discarding contaminated autoclavable and disposable items.

- ✓ Basically all specimens and used disposables should be autoclaved when finished with. Whether in the laboratory or in the field, these should have been collected into autoclavable bags or other suitable containers which are then autoclaved at 121 °C for ≥ 1 hour, preferably followed by incineration.
- ✓ Contaminated autoclavable non-disposable items should also be deposited in autoclavable containers and ultimately autoclaved.
- ✓ Microscope slides, coverslips and other sharp items should be placed in autoclavable sharps containers and autoclaved, preferably followed by incineration.
- ✓ There may be circumstances where it is appropriate to immerse items in hypochlorite solution (10 000 ppm) initially and then to autoclave and incinerate them later.
- ✓ Disinfect or fumigate non-autoclavable materials

Annex XII: photo of dried meat and ear clip collected samples

Left: Tissue sample (Dried meat) and Right: Ear clipping of anthrax suspected samples.

Annex XIII: Photos of different laboratory activities in Biosafety Level- III

Annex XIV: Anthrax suspected deaths in hippopotamus but negative for *B. anthracis* in both culture and real-time PCR

Death of hippopotamus at Ghibe sheleko national park suspected anthrax and collections of samples.

Annex XV: Anthrax suspected deaths in Afar Region in sheep and goat but negative for *B. anthracis* in both culture and real-time PCR

Anthrax suspected death of sheep and goat in Afar region and team collecting samples

Annex XVI: Disease outbreak investigation form

Date: ________________________

1. Name of Farm Owner: ----------------------------
2. Address: Region: -------------------- Zone:---------------------- District:--------------
   Kebele: ------------------------ Village:-------------------------------
3. Geographical location:
   Longitude: --------------------Latitude:----------------------Altitude:----------------
4. Type of Farm:
   Intensive---------------------- Extensive: -------------------------------------- Other:
5. Animals affected:
   1. Species: Bovine/ Ovine/ Caprine, / Equine/Wild animals
   Age: ----------Sex group: ---------Number at Risk: ------------------
   Number of Cases: ----------------number of Deaths: ---------------

6. History of the disease
   6. 1.Has anthrax occurred in this herd before? A. Yes B. No,
   6.2. If yes, when? A. in the last year B. in the last 10 years C. >10 years ago
   6.3. Has anthrax occurred in this site before? Yes/No,
   6.4. If yes, in the last year/ in the last 10 years/ >10 years ago
   6.5. Was the animal/herd in contact with, or located near another herd? Ans: Yes /No
   6.6. Is there any reason to suspect the feed? Ans: Yes/No
   If Yes, Year----------- Month----------
   What is the local name of the anthrax disease in your locality? ---------------------
   What are the clinical signs of anthrax of animals in the area?

   ----

7. Community practices of relevance for anthrax in the area they lived

7.1. What is your practices when animals dead from suspected anthrax cases

A. Reported to vets/ B. Sold meat, C. Ate meat D. Used for animal feeding

7.2. What is your participation when animal dies suddenly in community?

A. Skinning B Butchering C Cooking

7.3. Knowledge about proper carcass disposal

a. What do you do after death of the animal? A. Free grazing areas B. float in water C. bury in to the ground D. burning E. Forest and protected F. Agriculture G. Waste disposal

b. If buried, what will be its depth? A.1 meter B. 2 meter C. >2meter

Is the disease treatable? A. Yes B. No. If Yes, with what or how do you treat? ……………………………………………………………………………………………

8. Vaccination history:

Do you vaccinate your animals? Ans: Yes/No

If yes, when you vaccinate? ----------------------------Target population------------------

Number of animals vaccinated: ---------------------------- vaccination frequency? 6month/Annually

9. Does anthrax affects people in your area? A. Yes B. No
10. If yes how many human cases do you know? --------------------------------------
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11. Do you treat human cases? A. Yes B. No
12. With what do you treat?