PREVALENCE OF *Salmonella* AND *Shigella* Sp. IN READY-TO-EAT GAME MEAT SOLD IN DIFFERENT CITIES IN SOUTHWESTERN NIGERIA

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD

OF BACHELOR OF SCIENCE (B.Sc. Hons.) IN MICROBIOLOGY.

SEPTEMBER, 2022.

DECLARATION

I hereby declare that this project has been written by me and is a record of my own research work. It has not been presented in any previous application for a higher degree of this or any other University. All citations and sources of information are clearly acknowledged by means of reference.

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OLUWATOYIN, JOSHUA OLUWATOSIN

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Date

CERTIFICATION

This is to certify that the content of this project entitled **'PREVALENCE OF *Salmonella* AND *Shigella* Sp. IN READY-TO-EAT GAME MEAT SOLD IN DIFFERENT CITIES IN SOUTHWESTERN NIGERIA'** was prepared and submitted by **OLUWATOYIN, JOSHUA OLUWATOSIN** in partial fulfilment of the requirements for the degree of **BACHELOR OF SCIENCE IN MICROBIOLOGY.**

The original research work was carried out by him under my supervision and is hereby accepted.

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DEDICATION

I dedicate this research project to God Almighty who has been my sole source of inspiration, strength and wisdom. I also dedicate this project to my mother, (Mrs Oluwabunmilofe Sanusi) who has been my source of motivation.

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TABLE OF CONTENTS

Title Page……..……………………………………………………………………………….i

Declaration ii

Certification iii

Dedication iv

Acknowledgement v

List of Tables ix

List of Figures x

List of Plates xi

Abbreviations xii

Abstract xiii

CHAPTER ONE 1

INTRODUCTION 1

1.1 Background to the study 1

1.2 Statement of the problem 3

1.3 Justification of the study 3

1.4 Aim of the study 3

1.5 Objectives of the study 3

CHAPTER TWO 4

LITERATURE REVIEW 4

2.1 What is Game meat? 4

2.2 Implication of Game meat in Spread of Diseases 7

2.3 *Salmonella* sp. 9

2.3.1 Background History 9

2.3.2 Pathogenecity of *Salmonella* 9

2.3.3 Classification of *Salmonella* 10
2.3.3.1 Nontyphoidal *Salmonella* 10
2.3.3.2 Typhoidal *Salmonella* 11
2.3.5 Epidemiology of *Salmonella* 13
2.4 *Shigella* Sp. 15
2.4.1 Background History 15
2.4.2 Classification of *Shigella* 16
2.4.4 Pathogenicity of *Shigella* 18
2.4.5 Epidemiology of *Shigella* 20
2.5 Treatment of *Salmonella* and *Shigella* sp. Infections 20
2.6 Antibiotic resistance in *Salmonella* and *Shigella* sp. 21
CHAPTER THREE 23
MATERIALS AND METHODS 23
3.1 Sample Collection 23
3.2 Apparatus and Equipment 23
3.3 Media and Reagents 23
3.4 Preparation of Culture Media 25
3.4.1 Buffered Peptone water 25
3.4.2 Rappaport-Vassiliadis Soya Peptone Broth 25
3.4.3 Xylose Lysine Deoxycholate (XLD) Agar 26
3.4.5 Brain Heart Infusion Broth (BHI) 27
3.5 Isolation of *Salmonella* and *Shigella* Sp. 27
3.5.2 Secondary Enrichment 27
3.5.3 Plating Method 27
3.5.4 Pure Culture Technique 27

3.5.5 Cryopreservation of Isolates 28

3.6 Biochemical Test 28

3.6.1 Grams Staining 28

3.6.2 Catalase Test 28

3.6.3 Oxidase Test 29

3.6.4 Motility Test 29

3.6.5 Triple Sugar Iron Test 29

3.7 Molecular Identification 30

3.7.1 Activation of Isolates 30

3.7.2 DNA Extraction 30

3.7.3 Polymerase Chain Reaction (PCR) 31

3.7.4 Agarose Gel Electrophoresis 36

3.8 Precautions 36

CHAPTER FOUR 37

RESULTS AND DISCUSSION 37

4.1 Results 37

4.2 Biochemical Test of *Salmonella* sp 37

4.3 Biochemical Test for *Shigella* sp. 39

4.4 Molecular Identification of isolates 43

4.5 Discussion 47

CHAPTER FIVE 49

CONCLUSIONS AND RECOMMENDATIONS 49

5.1 Conclusions and Recommendations 49

REFERENCES 50

APPENDICES 67

LIST OF TABLES

TABLE	TITLE	PAGE
Table 2.1	List of common game meat species	6
Table 2.2	*Salmonella* sp. serovars isolated from wild game in European countries, 2000–2010	8
Table 2.3	Global Burden of Salmonellosis	14
Table 2.4	Classification of *Shigella* species	17
Table 3.1	Various game meat sampling locations in Nigeria	24
Table 3.2	Protocol for thermal cycler	32
Table 3.3	Forward and Reverse primers used for *Salmonella* subspecies 1gene amplification	33
Table 3.4	Components of PCR for *Salmonella* subspecies 1	34
Table 3.5	PCR reaction components used for 16s rRNA amplification	35
Table 4.1	Biochemical Test results of the presumptive *Salmonella* isolates	38
Table 4.2	Biochemical test results of the presumptive *Shigella* isolates	40
Table 4.3	Prevalence of *Salmonella* and *Shigella* sp. in the investigated game meat samples	45

LIST OF FIGURES

FIGURE	TITLE	PAGE
Figure 2.1	Classification of *Salmonella* Species and Subspecies	12
Figure 2.2	Infectious Cycle of *Shigella* involving the destruction of the colonic epithelium	19
Figure 4..1	Samples positive for *Salmonella* out of the total different game meat samples obtained	41
Figure 4.2	Samples positive for *Shigella* out of the total different game meat samples obtained	42

LIST OF PLATES

PLATE	TITLE	PAGE
Plate 4.1	Illustrative agarose gel electrophoresis image of a Simplex PCR assay of game meat samples for detecting the *Salmonella enterica* subsp. I (SSI) gene (137 bp)	44
Plate 4.2	Illustrative agarose gel electrophoresis image of PCR amplified products of 16 rRNA strains	45

ABBREVIATIONS

BHI – Brain Heart Infusion

BPW – Buffered Peptone Water

CDC – Centres for Disesease Control and Prevention

EHEC – Enterohemorrhagic *Escherichia coli*

HEA – Hektoen Enteric Agar

HIV – Human Immunodeficiency Virus

PCR – Polymerase Chain Reaction

RVS – Rappaport-Vassiliadis

SARS – Severe Acute Respiratory Syndrome

Sp – Species

TSI – Triple Sugar Iron

RTE – Ready to Eat

WHO – World Health Organization

XLD – Xylose Lysine Deoxycholate

ABSTRACT

Different meat products have been implicated in outbreaks of *Salmonella* and *Shigella* sp. worldwide. Game meat refers to any animal gotten from wildlife. A variety of game meat such as Grasscutters and Antelopes are predominantly consumed. However, hunting and consumption of game meat increases the risk of zoonotic infections. Also the process of hunting and processing wild animals is usually done under unsanitary situations in which they come into touch with humans and domesticated animals considerably increasing the chances of disease transmission and infection. This research was carried out to investigate the presence of *Salmonella* and *Shigella* species in different game meat sold in various cities within southwestern Nigeria. Isolation and identification of these two pathogens was performed using culture techniques and molecular techniques respectively, the isolates were then subjected to biochemical testing. The *Salmonella* isolates were confirmed by the presence of *Salmonella enterica* subsp. I (SSI) gene and the *Shigella* isolates were confirmed by the presence of 16s rRNA gene using simplex PCR. A total of 55 samples were analyzed for the presence of *Salmonella* and *Shigella* sp., 41.8% of the game meat samples investigated were positive for *Salmonella enterica* subspecies I and 45.5% were positive for *Shigella* sp. This high prevalence is of public health concern due to the low infective dose of these pathogens.

Keywords: Game meat, Public health, *Salmonella*, *Shigella,* Zoonosis.

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Foodborne gastroenteritis caused by enteric bacterial pathogens in human remains a major public health problem worldwide (Tadesse *et al.,* 2019). According to a report by the Centers for Disease Control and Prevention (CDC), it's been estimated that 48million illnesses, 128000 hospitalizations, and 3000 deaths occur each year due to foodborne diseases in the United States (Scallan *et al.,* 2011). Also in Africa it has been estimated that about 700000 deaths per year were caused due to foodborne and waterborne diseases (Mensah *et al.,* 2012). The issue of foodborne diseases is exacerbated in countries, where lack of food handling practices, inadequate food safety laws, weak regulatory systems, lack of financial resources to invest in safer equipment, and lack of food safety training are prevalent (Senthilkumar *et al.,* 2014; Tadesse *et al.,* 2019).

Salmonellosis is a disease caused by *Salmonella* sp. that affects both humans and animals worldwide. Although the disease typically manifests as mild gastroenteritis in humans, life-threatening systemic infections are very common, particularly among high-risk groups (LaRock *et al.,* 2015). Due to a major lack of coordinated national epidemiological surveillance systems and a poor healthcare system, the sources and routes of transmission of non-typhoidal *Salmonella* remain unknown throughout Africa (Gunn *et al.,* 2014). *Salmonella* is one of the primary sources of infection in food-producing animals, particularly chicken, and this has a direct impact on the global marketing of food-producing animals as well as animal-derived food items (Fagbamila *et al.,* 2017). *Salmonella* has been found in retail items such as chicken. *Salmonella* is most commonly seen in retail chicken (Li *et al.,* 2020). The use of possibly contaminated water during processing, as well as the quality of food packaging materials, may expose foods to pathogenic bacteria (Ehuwa *et al.,* 2021).

Shigellosis, commonly known as bacillary dysentery, is a bacterial infection caused by *Shigella* (Cetinkaya *et al.,* 2008). *Shigella sonnei* (about 70%) and *Shigella flexneri* (roughly 25%) are the most commonly implicated species, while other species are rarely implicated (Leting *et al.,* 2022). Due to their low infectious dosage, *Shigella* species have the potential to create huge outbreaks

(10 - 100cells) (Zaidi and Estrada-García, 2014). This disease can be transmitted through foods and drinking water (Nygren *et al.,* 2013). Each year, a large number of shigellosis outbreaks occur as a result of contaminated food intake (Nygren *et al.,* 2013). *Shigella* is one of the most common bacterial causes of diarrhea worldwide, according to the World Health Organization (WHO), there are around 165 million cases of severe dysentery worldwide. Every year, a million people die as a result of the disease. Mostly among children in developing countries (WHO, 2005).

According to the World Health Organization (WHO), Seventy-five percent of all the occurrence of infectious diseases in the last decade have originated in animals (WHO, 2020), Outbreaks of diseases such as Ebola, HIV, and SARS have been linked to the wild or game meat trade, with COVID-19 also potentially spread through this activity and causing devastating health and economic impacts (WILDAID, 2021). Nigeria has thriving game meat markets all around the country that offer both legal and illegal game meat. This trade is still unregulated. The process of hunting and processing wild animals under stressful and unsanitary situations in which they come into touch with humans and domesticated animals considerably raises the chances of disease transmission and infection (Gill, 2007; Paulsen *et al.,* 2012). Zoonosis, or animal-borne diseases linked to wildlife hunting and consumption, have emerged as a major source of concern around the world (Cantas and Suer, 2014). A survey of rural Nigerians found that 55% of respondents were aware of zoonoses. However, their education and cultural traditions are major drivers for hunting and eating gamemeat despite the risks involved (Friant *et al.,* 2015).

Game meat contamination can occur through the hunting process, slaughtering and processing stages including washing with water that has been contaminated as well as cross-contamination of ready-to-eat game meat (Sofos, 2014). Across the globe, outbreaks of illness linked to game meat have been connected to a wide range of bacteria, viruses and protozoa (Rahman *et al.,* 2020). Indeed, enteropathogenic bacteria such as enterohemorrhagic *Escherichia coli* (EHEC) and *Salmonella enterica,* whose principal reservoirs are food producing animals, are well-known sources of concern for the safety of fresh meat and poultry (Rhoades *et al.,* 2009). The pathogenic bacteria *Listeria monocytogenes*, on the other hand, has been identified as a pathogen of concern in ready-to-eat (RTE) beef and poultry items that have been exposed to postprocessing contamination and are promoting the organism's growth during storage (USFDA/USDA-FSIS, 2003). Nonetheless, additional bacterial species such as *Campylobacter jejuni, Clostridium*

perfringens, and *Yersinia enterocolitica* may provide meat safety concerns, as might viral infections, parasites, and other biological challenges like as prions (Lianou *et al.,* 2017).

Salmonella and *Shigella* sp. have been found from previous studies to be a part of the aetiological agents of infections related to game meat (Gill, 2007; Rhoades *et al.,* 2009; Bachand *et al.,* 2012; Guerra *et al.,* 2016). Although several studies have been conducted on salmonellosis and shigellosis, there is limited information about the occurrence of *Salmonella* and *Shigella* sp. in Game meat. This study was conducted to determine the prevalence of *Salmonella* and *Shigella* sp. in ready to eat game meat.

1.2 Statement of the problem

Game meat safety is inextricably tied to the hunting process, as well as the cleanliness and temperature of carcasses as they are handled and transported. Game meat brings humans into close contact with wildlife, facilitating the spread of foodborne and viral diseases such as Ebola and diarrhea as well as new developing infectious diseases. The spread of these diseases could be wider from the consumption of unsafe game meats that are potential reservoirs of the causative pathogens.

1.3 Justification of the study

It is crucial to investigate the microbiological safety of game meat in order to ascertain the level of pathogens present in it and identify the most prevalent organisms. Various samples of game meat from different locations in Nigeria would be examined for the presence of *Salmonella* and *Shigella* sp. which if present poses the risk of morbidity and mortality associated with the consumption of contaminated game meat.

1.4 Aim of the study

The aim of the study is to investigate the prevalence of *Salmonella* and *Shigella* sp. in ready-to-eat game meat sold in different cities in southwestern Nigeria.

1.5 Objectives of the study

- To isolate *Salmonella* and *Shigella* sp. in ready-to-eat game meat sold in the various market areas of Nigeria using cultural methods.
- To identify isolated *Salmonella* and *Shigella* sp. using molecular techniques.

CHAPTER TWO

LITERATURE REVIEW

2.1 What is Game meat?

Game meat refers to meat from wildlife species that are hunted for human consumption, and is most commonly associated with game meat in Africa. Game meat is a major source of animal nutrition as well as a cash crop for people living in humid tropical forest regions of Africa, Latin America, and Asia (Nasi *et al.,* 2008). The term 'game meat' comes from an African term for animal species that are hunted for human consumption (Bennett *et al.,* 2007), and it mainly refers to African wildlife meat (Hall, 2019). Wildlife hunting for food is critical for poor people's livelihood security and nutritional protein supply. When done out by traditional hunter-gatherers in wide expanses for their own consumption, it can be sustainable. Game meat is a good source of vitamins and macronutrients. A study of South Americans in the Tres Fronteras region discovered that individuals who ate game meat had a decreased incidence of anemia and chronic health problems because their diets contained more iron, zinc, and vitamin C than those who did not eat game meat (Lee *et al.,* 2020).

According to a survey carried out on 2000 people by Wildaid between september and october 2020 across four major cities (800 in Lagos, 450 in Abuja, 450 in Port Harcourt and 300 in Calabar) in Nigeria, it was observed that In Nigeria, consumption of game meat is predominant; 71 percent of respondents reported having done it at some point in their life, with 45 percent having done so in the previous year. One in sixteen people who have eaten bush meat in the past year do so on a weekly basis. Nearly half of consumers (47%) claim that their parents had an influence on their decision to consume, while 40% claim that they were the ones who made the choice. In Nigeria, the majority of game meat consumers (44%) eat grasscutters, which are then followed by antelope/deer (25%) snake (21%) and wild pig (15%). Other animals eaten include monkeys (11%), porcupines (10%), tortoises (9%), crocodiles (8%) and monitor lizards (7%) as well as bats (6%) and sea turtles (4%) and chimpanzees, pangolins, hedgehogs, and civets (approximately 2 percent each). The consumption of other game meat species besides those mentioned above was reported by 32% of consumers of game meat. These may also include animals such the genet, squirrel, giant rat, rock hyrax, guinea fowl, mongoose, and buffalo, which are frequently seen in game meat markets. 51 percent of customers who had consumed game meat within the previous

year think it is more delicious than farmed meat, and 30 percent see it as an important aspect of their culture. Up to 28% think it is more nutritious and fresh than commonly seen domestically farmed meat and fish because it contains less chemicals (WILDAID, 2021).

Table 2.1 List of common game meat species (WILDAID, 2021).

NAME	SPECIES
Grasscutter	Nil
Antelope	Western hartebeest, red-flanked duiker, bay duiker, Maxwell's duiker, black duiker, Ogilby's duiker, yellow-backed duiker, Dwarf antelope, topi, roan antelope, waterbuck, kob, klipspringer, oribi, Bohor reedbuck
Snake	Royal python, rock python
Wild Pig	Red river hog
Monkey	Olive colobus, Niger Delta red colobus, Preus's red colobus, red-capped mangabey, drill, whitethroated monkey, Sclater's guenon
Porcupine	Crested porcupine
Tortoise	African spurred tortoise, Senegal flapshell turtle, serrated hinge-back tortoise
Crocodile	Nile crocodile, slender-snouted crocodile, dwarf crocodile
Monitor Lizard	Nile monitor lizard, Bosc's monitor lizard
Bat	Straw-colored fruit bat, Franquet's epauletted fruit bat, hammer-headed bat, Egyptian rousette fruit bat
Sea Turtle	Hawksbill turtle, Olive ridley, green sea turtle, leatherback turtle,
Chimpanzee	Nigeria-Cameroon chimpanzee
Pangolin	Giant ground pangolin, white-bellied tree pangolin, long-tailed (black-bellied) pangolin
Hegdehog	Nil
Civet	African civet, African palm civet

2.2 Implication of Game meat in Spread of Diseases

Game meat is derived from a range of wild animals, including bats, nonhuman primates (monkeys), cane rats (grasscutters), and duiker (antelope). Game meat is frequently smoked, dried, or salted (these procedures are not sufficient to render the meat noninfectious). People may have contracted infectious diseases such as tuberculosis, leprosy, cholera, smallpox, measles, influenza, and syphilis from animal sources. Today, animal sources are blamed for the advent of HIV-1, AIDS, Ebola virus disease, and Creutzfeldt-Jakob disease (McMichael, 2002). In the 1980s, Thomas's rope squirrel *(Funisciurus anerythrus)* and red-legged sun squirrel (*Heliosciurus rufobrachium*) were identified as monkeypox virus reservoirs in the Democratic Republic of the Congo (Khodakevich *et al.,* 1987). Ebola outbreaks in the Congo Basin and Gabon in the 1990s were linked to the killing and consumption of chimps and bonobos (Georges-Courbot *et al.,* 1997). When butchering and eating ungulates, anthrax can be passed on. When butchering a carcass, the risk of spreading bloodborne infections is higher than when transporting, cooking, and eating it (Wolfe *et al.,* 2005). Many hunters and traders are unaware of the dangers of zoonosis and the risk of disease transmission (Subramanian, 2012).

In Cameroon, 15 primate species were tested for parasites in their gastrointestinal tracts. *Trichuris, Entamoeba, Ascaris, Capillaria, pinworms, Bertiella*, and *Endolimax nana* were found in game meat primates (Pourrut *et al.,* 2011). In the Democratic Republic of the Congo, a substantial percentage of *Bitis vipers* sold in local game meat markets are infected with *Armillifer grandis*, posing a public health risk (Hardi *et al.,* 2017).

According to Paulsen *et al.,* (2012) *Salmonella enterica* serovars has been isolated, with *S. Typhimurium* and *S. Enteritidis* having the highest prevalences. It has been acknowledged in the framework of zoonoses legislation that the persistence of these pathogens in wildlife is a less urgent matter for biology and wildlife conservationists than for public health professionals (Paulsen *et al.,* 2012).

Table 2.2 ***Salmonella*** **sp. serovars isolated from wild game in European countries, 2000–2010**

Type of animal	Country	Sample	*S. enterica* serovars	References
Wild ruminants	Italy	Feces	*Typhimurium, Napoli, Veneziana, Mishmarhaemek*	(Magnino *et al.*, 2011).
	Austria	Carcass	*Enteritidis*	(Deutz *et al.*, 2000).
Wild boar	Switzerland	Tonsils, feces	*Enteritidis, Veneziana, Stourbridge*	(Wacheck *et al.*, 2010)
	Portugal	Feces	*Typhimurium, Rissen*	(Vieira-Pinto *et al.*, 2011).
	Italy	Feces	*Coeln, Typhimurium, Ball, Thompson, Veneziana, Enteritidis, Infantis; S. enterica diarizonae*	(Magnino *et al.*, 2011).
	Germany	Lymph node	*Hadar*	(Ziegenfuss, 2003)
	Poland	Carcass	*Bardo*	(Wisniewski, 2001)
Wild rabbit	Portugal	Feces	*Rissen, Enteritidis, Havana, Typhimurium, Derby*	(Vieira-Pinto *et al.*, 2011)
Pheasant	Germany	Feces	*Typhimurium*	(Backhus, 2000)

2.3 *Salmonella* sp.

2.3.1 Background History

Salmonella is a Gram-negative bacteria genus of rod-shaped (bacillus) bacteria in the *Enterobacteriaceae* family. *Salmonella enterica* and *Salmonella bongori* are the two types of *Salmonella*. The type species is *S. enterica*, which is divided into six subspecies (Su and Chiu, 2007; Ryan *et al.,* 2017) which include over 2,600 serotypes (Gal-Mor *et al.,* 2014). *Salmonella* was named after American veterinary surgeon, Daniel Elmer Salmon (1850–1914). *Salmonella* species are non-spore-forming, mostly motile enterobacteria with cell diameters between about 0.7 and 1.5 µm, lengths from 2 to 5 µm, and peritrichous flagella (all around the cell body, for movement) (Fàbrega and Vila, 2013). They are chemotrophs, meaning they get their energy from organic sources through oxidation and reduction reactions. They're also facultative anaerobes, meaning they may generate ATP using oxygen ("aerobically") when it's accessible, or other electron acceptors or fermentation ("anaerobically") when oxygen is not available (Fàbrega and Vila, 2013).

Salmonella is one of the most common pathogenic bacteria implicated in foodborne outbreaks and diseases (Cetinkaya *et al.,* 2008). *Salmonella* is further classified into typhoidal and nontyphoidal strains. *Salmonella enteritidis* and *Salmonella daviana* are examples of non-typhoidal *Salmonella*, which can be carried by both people and animals. *Salmonella typhi*, which is carried by humans and causes typhoid fever, is the cause of typhoid fever (Gal-Mor *et al.,* 2014). Diarrhoea, stomach pains, fever, and vomiting are some of the infection's symptoms (Kia *et al.,* 2020). Salmonellosis can be transmitted in a variety of ways, but the majority of human infections are caused by consumption of contaminated foods, particularly those of animal origin (Hernandez *et al.,* 2005).

2.3.2 Pathogenecity of *Salmonella*

Salmonella has an abnormal activity during its invasion of non-phagocytic human host cells (HansenWester *et al.,* 2002), in which it promotes its own phagocytosis in order to obtain access to the host cell. *Salmonella* pathogenicity islands (SPIs), gene clusters located at the vast chromosomal DNA region and encoding for the structures needed in the invasion process, are the remarkable genetics underlying this brilliant technique (Grassl and Finlay 2008). Bacteria that enter the digestive tract from contaminated water or food tend to infiltrate the epithelial cells that line the intestinal wall. Type III secretion systems, multi-channel proteins that allow *Salmonella*

to inject its effectors through the intestinal epithelial cell membrane into the cytoplasm, are encoded by SPIs. The bacterial effectors subsequently activate the signal transduction pathway and cause the host cell's actin cytoskeleton to be reconstructed, resulting in the outward expansion or ruffling of the epithelial cell membrane to engulf the bacteria. The shape of the membrane ruffle mirrors the phagocytosis process (Takaya et al. 2003). *Salmonella* strains' ability to survive in the host cell is critical for pathogenesis, as strains lacking this property are non-virulent (Bakowski et al. 2008). Following *Salmonella* engulfment in the host cell, the bacteria is enclosed in a membrane compartment known as a vacuole, which is made up of the host cell membrane (Shu-Kee *et al.,* 2015).

2.3.3 Classification of *Salmonella*

2.3.3.1 Nontyphoidal *Salmonella*

There are approximately 2,000 nontyphoidal *Salmonella* serotypes known, which are thought to be responsible for up to 1.4 million illnesses in the United States each year. Infants, the elderly, organ transplant recipients, and the immunocompromised are all at risk for serious illness (Goldrick and Barbara, 2003). While non-typhoidal serotypes are usually associated with gastrointestinal sickness in affluent nations, they can cause serious bloodstream infections in Sub-Saharan Africa and are the most commonly isolated bacterium from the blood of people who have a fever. In 2012, a case fatality rate of 20–25 percent was observed in Africa from nontyphoidal *Salmonella*e bloodstream infections. *Salmonella enterica Typhimurium* or *Salmonella enterica Enteritidis* are the most common causes of invasive nontyphoidal *Salmonella* infection (iNTS). A new strain of *Salmonella Typhimurium* (ST313) appeared 75 years ago in the southeast of Africa, followed by a second wave from central Africa 18 years later. This second wave of iNTS may have started in the Congo Basin and acquired a gene that made it resistant to the antibiotic chloramphenicol early on. This necessitated the use of costly antimicrobial drugs in impoverished areas of Africa, making treatment difficult. The high frequency of iNTS in Sub-Saharan Africa compared to other locations is assumed to be attributable to the huge proportion of the African population suffering from immunological suppression or impairment as a result of HIV, malaria, and malnutrition, particularly among children. The genetic makeup of iNTS is evolving, and it is becoming more typhoid-like, with the ability to spread quickly throughout the human body. Fever,

hepatosplenomegaly, and respiratory symptoms have all been documented, along with the absence of gastrointestinal symptoms (Feasey *et al.,* 2012).

2.3.3.2 Typhoidal *Salmonella*

Salmonella serotypes that are strictly suited to humans or higher primates, such as *Salmonella Typhi*, *Paratyphi A, Paratyphi B*, and *Paratyphi C,* cause typhoid fever (Näsström *et al.,* 2014). *Salmonellae* enter the bloodstream through the lymphatic system of the colon (typhoid form) and are transferred to various organs (liver, spleen, kidneys) to establish secondary foci in the systemic form of the disease (septic form) (Gunn *et al.,* 2014). Endotoxins first affect the vascular and neurological systems, causing increased permeability and decreased vessel tone, as well as a disruption in temperature regulation, vomiting, and diarrhoea (Sanchez, 2018). In extreme cases, enough liquid and electrolytes are lost to disrupt water-salt metabolism, lower circulating blood volume and arterial pressure, and result in hypovolemic shock. Septic shock is also a possibility (Taghavi *et al.,* 2022). In severe salmonellosis, mixed shock (with indications of both hypovolemic and septic shock) is more likely. Renal involvement owing to hypoxia and toxaemia can cause oliguria and azotemia in severe situations (Peerapornratana *et al.,* 2019).

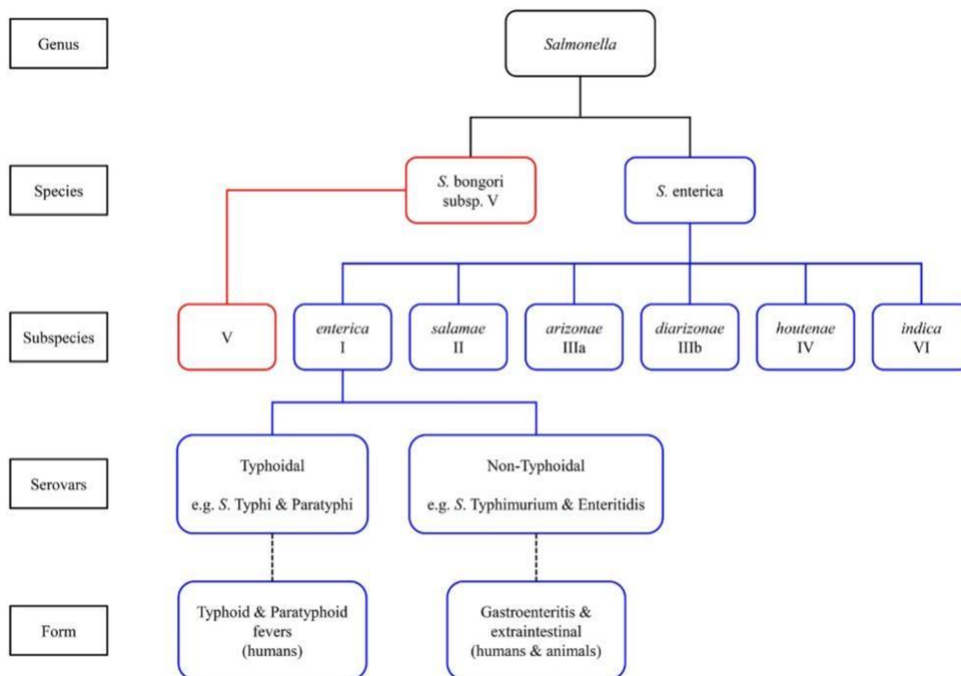

Figure 2.1: Classification of *Salmonella* Species and Subspecies (Hurley *et al.,* 2014).

2.3.5 Epidemiology of *Salmonella*

Typhoid cases have been constant and low in affluent countries, whereas non-typhoidal salmonellosis has increased globally. Typhoid fever frequently kills 5 to 30% of typhoid-infected people in the underdeveloped nations. According to the World Health Organization (WHO), 16 to 17 million cases occur each year, resulting in approximately 600,000 deaths. Despite the use of adequate antibiotic treatment, mortality rates vary by region and can be as high as 5 to 7%. (Scherer and Miller, 2001; Nwabor *et al.,* 2015). Typhoid fever is one of the most common diseases in Nigeria, affecting both young children and young adults as a result of a number of interconnected variables such as poor facilities for processing human waste and indiscriminate antibiotic usage. Morbidity related with *Salmonella* disease is on the rise, with some cases leading to death (Akinyemi *et al.,* 2005). A more exact figure for salmonellosis is difficult to determine because only significant outbreaks are often investigated, whereas sporadic cases are under-reported (Scherer and Miller, 2001; Parry, 2006). Non-typhoidal cases, on the other hand, account for 1.3 billion cases and 3 million deaths (Hanes, 2003; Hu and Kopecko, 2003).

In their assessment on the global impact of non-typhoidal *Salmonella* invasive disease, Stanaway *et al.,* (2019) stated that non-typhoidal *Salmonella* continues to be a major source of disease and death globally. Greater risk group include malnourished young children, the elderly, immunocompromised individuals (such HIV patients), people who have acute malaria, and people who already have a chronic illness. In addition to diarrhoea, this infection can affect healthy hosts and result in bacteraemia, meningitis, and tonsil infections. According to The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) (Stanaway *et al.,* 2019), *Salmonella enterocolitis* caused 95.1 million disease conditions, 3.1 million disability-adjusted life-years, and 50,771 fatalities in 2017. In 2010, the Foodborne Disease Burden Epidemiology Reference Group (FERG) of the WHO reported that a total of 180M illnesses and 298,496 deaths were attributed to *Salmonella.*

Table 2.3 Global Burden of Salmonellosis

***Salmonella* Serovars**	**Illnesses**	**Death**	**References**
S. enterica, non-typhoidal	153,097,991	56,969	(WHO, 2015).
Invasive non-typhoidal *S. enterica*	596,824	63,312	(WHO, 2015).
Invasive non-typhoidal *S. enterica*	535,000	77,500	(Stanaway *et al.,* 2019)
S. enterica Paratyphi A	4,826,477	33,325	(Kirk *et al.,* 2015)
S. enterica Typhi	20,984,683	144,890	(Kirk *et al.,* 2015)

The majority of disease burden was caused by invasive non-typhoidal *S. enterica* foodborne infections. This is because the bacterium is widespread, it frequently infects children, and it causes severe diarrhoea (Majowicz *et al.,* 2014). *Salmonella* serotype Y poses the greatest foodborne burden, according to Kirk *et al.*'s assessment of the health effects of all *Salmonella* serotypes. Combining information related to *S. enterica* from diarrheal illnesses and invasive Non- Typhoidal *Salmonella* (iNTS), *Salmonella Typhi*, and *Salmonella Paratyphi A* infections, a total of 8.76 million Disability-Adjusted Life Years (DALY) from all sources of transmission and 6.43 million linked to contaminated foods were recorded (Kirk *et al.,* 2015).

2.4 *Shigella* Sp.

2.4.1 Background History

Shigella is a genus of gram-negative, facultative anaerobe, non-spore forming, non-motile, rod-shaped bacteria that have a close genetic relationship with *E. coli*. The genus was named after Kiyoshi Shiga who discovered it in 1897 (Shad and Shad, 2021). *Shigella* is the pathogen that causes human shigellosis, although it does not affect other mammals. *Shigella* only affects primates (Dekker and Frank , 2015). Only gorillas and humans naturally carry it (Lampel *et al.,* 2018). Dysentery is frequently the result of infection (Bennish and Albert , 2011). With an estimated 80–165 million cases worldwide, *Shigella* is one of the most common germs to cause diarrhoea (Bowen, 2016). It is believed that between 74,000 and 600,000 people die from it each year (Bowen, 2016; Mani *et al.,* 2016). It is one of the top four infections responsible for moderate-to-severe diarrhoea in children from South Asia and Africa (Kotloff *et al.,* 2013).

Shigella sp. are predominantly transmitted by fecal-oral routes through person to person contact, contaminated food and water. Some other transmission route from polluted faecal waste has been identified. *Shigella* infections can also be spread among male homosexuals through oral-anal contact (Guillot and Loret, 2009). *Shigella* is typically thought to have a restricted range in nature, primarily inhabiting human intestinal tracts and captive primates where shigellosis naturally develops (Dekker and Frank, 2015). There is no evidence, however, that the disease naturally occurs, particularly in the wild in those monkeys, without prior contact with humans (Shipley *et al.,* 2010).

2.4.2 Classification of *Shigella*

Based on their O antigen, the four *Shigella* species (subgroups) are classified as serotypes and subserotypes. *S. dysenteriae* (subgroup A) has 15 serotypes, *S. fexneri* (subgroup B) has 18 serotypes, *Shigella boydii* (subgroup C) has 20 serotypes, and *S. sonnei* (subgroup D) has a single serotype (Levine *et al.,* 2007). Notably, *Shigella* sp. identification must be based on both serological and biochemical traits (Van *et al.,* 2012). Traditionally, serotyping was done with in-house or commercial antisera against LPS O-antigen, which are split into polyvalent and monovalent antisera. Polyvalent antisera include antibodies for many *Shigella* serotypes and can be used to determine *Shigella* subgroups, whereas monovalent antisera contain serotype-specific antibodies (Van *et al.,* 2012).

Table 2.4 Classification of *Shigella* species (Mattock and Blocker, 2017).

Serogroup	Species	Number of Serotypes
A	*dysenteriae*	15
B	*flexneri*	18
C	*boydii*	20
D	*sonnei*	1

2.4.4 Pathogenicity of *Shigella*

Shigella are capable of penetrating the bowel's epithelial tissue due to their invasive characteristics, and their toxin plays a crucial role in pathogenesis (Lampel *et al.,* 2018). The infectious dose is negligibly low. *Shigella* usually has a low infectious dose, but when ingested, it can cause an intense watery or mucoid/bloody diarrhea. This clinical manifestation is a direct result of *Shigella* invasion and extensive intestinal epithelial damage. In a nutshell, the bacterium uses M cells to get through the epithelium and trigger macrophage phagocytosis in the submucosa. *Shigella* rapidly activates macrophage death and interacts with the basolateral surface of the epithelium, causing its absorption by causing the cytoskeleton of the host cell to reorganize (Zychlinsky *et al.,* 1992; Baker and The, 2018). As soon as they are inside the epithelial cell, they reproduce and once more lyse the nearby phagosome before spreading intracellularly to other cells through actin polymerization (Baker and The, 2018). The enormous virulence plasmid (more than 200 kbp), which encodes the syringe-like type three secretion system (T3SS) and a plethora of effector proteins, including multiple invasion plasmid antigens (Ipas), is essential to this well choreographed pathogenesis (Venkatesan *et al*, 2001). Numerous reviews have been written to explain the pathophysiology of *Shigella* and to clarify the function of each virulence component (Mattock and Blocker, 2017).

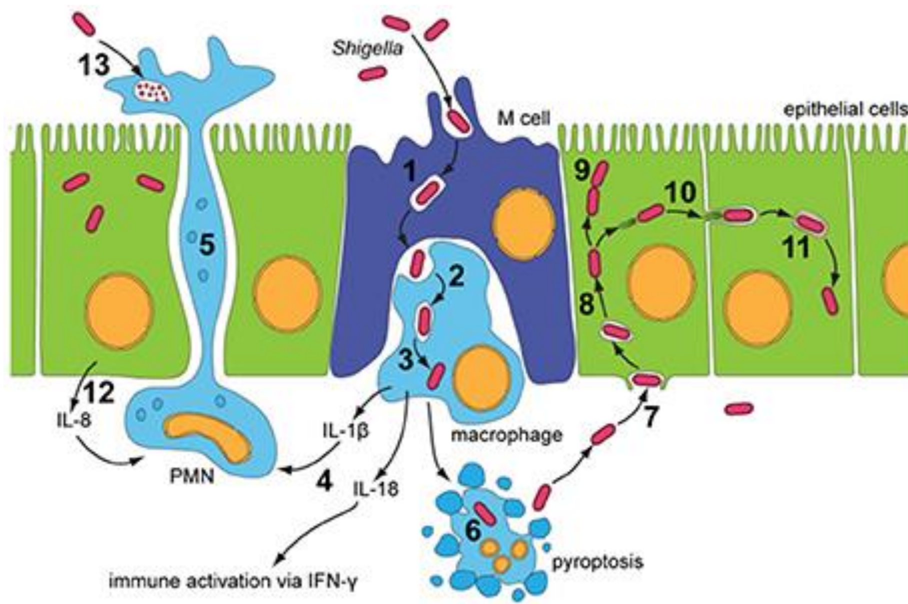

Figure 2.2: Infectious Cycle of *Shigella* involving the destruction of the colonic epithelium (Mattock and Blocker, 2017).

2.4.5 Epidemiology of *Shigella*

Diarrheal diseases are the leading worldwide cause of death among children. According to the World Health Organization, diarrheal illnesses are responsible for 5 million annual deaths (Hameed *et al.,* 2018), and *Shigella* is responsible for 10% of these deaths. Epidemic dysentery, which is typically severe and prolonged and is caused by *S. dysenteriae* 1 (the only *Shigella* sp. that produce Shiga toxins), is a persistent issue in many of the world's poorest regions, particularly in Africa, Central America, and some regions of Asia. Many of these outbreaks are caused by multiple antibiotic resistant strains; the deaths rate of these infections can be as high as 20%. In addition, *S. dysenteriae* 1 appears to develop antibiotic resistance more quickly than other *Shigella* sp. (Fratamico *et al.,* 2005). Most cases following person-to-person transmission. Usually, food and/or water pollution causes outbreaks (Guillot and Loret, 2009). The disease is endemic throughout the world, although 99% of the cases occur in developing countries. Shigellosis is a disease that affects the poorest populations of the planet. Bacillary dysentery is thought to affect 164.7 million people annually, of which 163.2 million live in developed nations and 1.5 million do so in industrialized nations. Shigellosis claims the lives of approximately 1.1 million people annually, 61% of whom are children under the age of five (Gillespie and Hawkey, 2006). Although widespread over the world, bacillary dysentery prevalence varies by location. Shigellosis outbreaks have always happened among people who gather in unsanitary settings, such as armies during military operations, pilgrimages, and refugee camps. Despite the abundance of epidemiological studies that are currently available, there is still a critical need for a precise and regularly updated assessment of the disease burden (Guillot and Loret, 2009).

2.5 Treatment of *Salmonella* and *Shigella* sp. Infections

In addition to significantly enhancing quality of life and lowering mortality from bacterial infections, antibiotics are essential in the treatment of infectious diseases. Antibiotic drugs' selectivity against invasive bacteria guarantees maximum eradication of the target microorganisms while causing the least amount of harm to people (Nami *et al.,* 2015). Typically, antibiotics are not needed to treat NTS infections. However, complications like meningitis and septicemia might occur and need to be treated with antibiotics such ciprofloxacin, ceftriaxone, and ampicillin (Medalla *et al.,* 2016). Using antibiotics such cefixime, chloramphenicol, amoxicillin, trimethoprim/sulfamethoxazole (TMP-SMX), azithromycin, aztreonam, cefotaxime, or ceftriaxone to treat *S. Typhi* and *S. Paratyphi* infections is necessary to prevent mortality (Kumar

et al., 2017). A corticosteroid medication called dexamethasone may be used when complications including delirium, obtundation, stupor, coma, or shock manifest (Kumar *et al.,* 2017). As soon as a susceptibility result is known, antibiotics should be modified. If established susceptibility exists, second-generation cephalosporin, ampicillin, and trimethoprim-sulfamethoxazole may also be used to treat *Shigella* infections.

If antibiotic susceptibility is unknown, azithromycin is the first-line treatment in children. A randomized experiment revealed that azithromycin was clinically and bacteriologically effective in 82% and 94% of patients, respectively (Aslam and Okafor, 2022). Due to the extensive resistance to widely-used antibiotics in South Asia, cefixime and ceftibuten can be used as the first-line treatment for shigellosis (Rahman *et al.,* 2007). The alternate treatment plan calls for the use of pivmecillinam, an extended-spectrum penicillin that works well at shortening the length of diarrhea and eliminating *Shigella* organisms from the stool (Traa *et al.,* 2010). Children with suspected or confirmed shigellosis who have a severe illness and symptoms of bacteremia, such as lethargy, a temperature more than 39° C (102.2°F), an underlying immunological deficiency, such as AIDS, or who are unable to take oral drugs should get parenteral antibiotics (Aslam and Okafor, 2022). It is advised to take ceftriaxone either once or five times daily.

2.6 Antibiotic resistance in *Salmonella* and *Shigella* sp.

Antibiotic resistance in foodborne organisms like *Salmonella* is a major public health concern. More attention is needed to target them in the animal food supply (CDC, 2013). *Salmonella* is difficult to eradicate from its reservoir hosts, therefore food animals are frequently used as reservoirs. Non-typhoidal *Salmonella* is responsible for the greatest number of foodborne infections, hospitalizations, and deaths (Scallan *et al.,* 2011). It causes more than 1,200,000 illnesses each year, with at least 100,000 of these infections caused by antibiotic-resistant *Salmonella*, including those resistant to clinically significant medications such ceftriaxone (36,000 illnesses per year) and ciprofloxacin (33,000 illnesses per year) (CDC, 2013). Two *S. flexneri* strains, *S. flexneri* 021787 and *S. flexneri* 021895, were found to be resistant to fluoroquinolones in a study conducted in Korea. (J.-Y. Kim *et al.,* 2008).

Shigella sp. are antibiotic-resistant, and drug treatment for these pathogens is expensive, time-consuming, and often troublesome, particularly in locations with poor medical care (Taneja and Mewara, 2016). Approximately 50% of *Shigella* strains in several regions of the world are now

resistant to numerous treatments (Ranjbar and Farahani, 2019). Researchers have recently described various antibiotic-resistance pathways, and these antibiotic-resistance mechanisms limit therapeutic options for *Shigella* infections (Qiu *et al.,* 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection

The sampling locations were Oyo, Osun, Ondo, Ogun and Lagos state. The southwestern part of Nigeria has been identified as some of the states with the highest numbers of game-meat consumers. Various game meat were purchased from various open markets as shown in Table 3.1. The purchased samples were placed in sterile food-grade bags and kept on ice packs in cooling boxes and then transported to the laboratory where they were immediately analyzed.

3.2 Apparatus and Equipment

Apparatus used include: stomacher bags, wash bottles, petri-dishes, measuring cylinder, glass pipettes, beakers, conical flasks, glass spreader, inoculating loop, wash bottles, eppendorf tubes, micro pipette (with their tips), test tubes and foil corks (with their racks), PCR tubes, glass slides, oxidase test disc.

Equipment used: Analytical balance, Hot plate stirrer, Autoclave, Vortex mixer, Water distiller, Water bath (set at 80°C), Incubator (37°C), Bunsen burner, Centrifuge, Heating block, Thermal cycler, Gel electrophoresis tanks, Gel documentation system, Microscope.

3.3 Media and Reagents

The work area was sterilized by using 70% ethanol to inactivate any microbial contaminants.

For isolation of *Salmonella* and *Shigella* species: 1% Buffered peptone water (BPW), Rappaport-Vassiliadis (RVS) Enrichment Broth (Merck, Darmstadt, Germany), Xylose Lysine Deoxycholate (XLD) Agar (Merck, Darmstadt, Germany), Hektoen Enteric Agar (HEA) (Liofilchem, Italy), Brain Hearth Infusion (BHI) Broth (Liofilchem, Italy), 20% Glycerol, Distilled water.

For molecular identification: Agarose, 1x TAE buffer, master mix, specific primers, Nuclease free water, Ethidium Bromide.

Table 3.1 Various game meat sampling locations in Nigeria

LOCATION	GAME-MEAT	NUMBER OF SAMPLES
	Pangolin	
	Bird	
	Deer	
	Bush dog	
	Grasscutter	
Lagos State	Etu	25
	Wild Cat	
	Atika	
	Agbonrin	
	Antelope	
	Monkey	
	Rabbit	
	Porcupine	
	Antelope	
	Grasscutter	
	Rabbit	
Ogun State	Bush rat	
	Igala	
	Hedgehog	12
	Guinea fowl	
	Alligator	
	Civet Cat	
	Rabbit	
	Antelope	
Ondo State	Grasscutter	9
	Grasscutter	
	Guinea Fowl	
	Hare	
	Sese	
Osun State	Antelope	5
	Aparo	
	Eta	
Oyo State	Esii Tuku	4
	Guinea Fowl	
Total		55

3.4 Preparation of Culture Media

3.4.1 Buffered Peptone water

Buffered Peptone Water is a microbiological growth medium made up of sodium chloride and peptic digest of animal tissue. The medium has a pH of 7.2 ± 0.2 at 25°C and is high in tryptophan. Buffered peptone water is a nonselective broth medium that can be used to grow bacteria as a primary enrichment media. The preparation of the media is as follows;

- 10g of the dehydrated medium was dissolved in 1litre of distilled water in a conical flask and was mixed thoroughly. The conical flask is then closed with a foil cork.
- The mixture was then stirred for a while using the magnetic stirrer hot plate to dissolve the powder completely.
- 225ml of the 1% was then dispensed into conical flasks.
- The conical flasks containing the media was then autoclaved at 121°C for 15mins.

3.4.2 Rappaport-Vassiliadis Soya Peptone Broth

Rappaport-Vassiliadis Soya Peptone Broth is recommended as a selective enrichment medium for the isolation of *Salmonella*e from food and environmental specimens (Rappaport *et al.,* 1956).The ability to exploit the full characteristics of *Salmonella* species when compared with other *Enterobacteriaceae*. These are:

- The ability to survive at relatively high osmotic pressure.
- To multiply at relatively low pH values.
- To be relatively more resistant to malachite green.
- To have relatively less demanding nutritional requirements.

Rapparport-Vassiliadis Soya Peptone Broth was prepared according to the instruction of the manufacturer.

- 26.75g was suspended in 1 litre of distilled water.
- The mixture was then stirred for a while using the magnetic rod stirrer hot plate to dissolve the powder completely.
- 9ml volumes of the dissolved mixture were then dispensed into test tubes.
- The test tubes containing the media were then autoclaved at 115°C for 15 minutes.

3.4.3 Xylose Lysine Deoxycholate (XLD) Agar

Xylose Lysine Deoxycholate (XLD) Agar is a selective medium for the isolation of *Salmonella* and *Shigella* sp. from clinical specimens and food samples. Taylor developed XLD Agar to isolate and identify *Shigella* from stool specimens. Pathogens are differentiated not just from non-pathogenic lactose fermenters, but also from many non-pathogens that do not ferment lactose or sucrose. Furthermore, the medium was designed to boost the frequency of development of the more fastidious pathogens, which have previously failed to thrive in other formulations due to the inclusion of extremely harsh inhibitors. A number of clinical studies have validated the assertion that XLD Agar has a reasonably high efficiency in the primary isolation of *Shigella* and *Salmonella* sp. XLD Agar is recommended for the testing of foods, dairy products and water. Preparation of the media is as follows;

- Suspend 55 grams of dehydrated medium in 1000 ml purified or distilled water.
- Heat with frequent agitation until the medium boils.
  Note: DO NOT AUTOCLAVE.
- Transfer immediately to a water bath at 50°C.
- After cooling, pour into sterile Petri plates.

3.4.4 Hektoen Enteric Agar (HEA)

Hektoen Enteric Agar (HEA) is a selective and differential medium used to isolate and differentiate *Salmonella* and *Shigella* species from other Enterobacteriaceae. Most Gram positive organisms are inhibited by bile salts and the dyes bromthymol blue and acid fuchsin. Lactose, sucrose, and salicin are fermentable carbohydrates that promote intestinal growth and differentiation. Sulfur can be obtained from sodium thiosulfate. Ferric ammonium citrate provides an iron source for the manufacture of hydrogen sulphide from sodium thiosulfate, which provides a sulphur source. By interacting with hydrogen sulphide gas to form a black precipitate, ferric ammonium citrate can also be used to visualise hydrogen sulphide production. Preparation of the media is as follows;

- Suspend 72.66 grams in 1000 ml purified/ distilled water.
- Heat to boiling to dissolve the medium completely.
- Note: DO NOT AUTOCLAVE.
- Allow to cool 45-50°C.
- Mix well and pour into sterile petri dishes.

3.4.5 Brain Heart Infusion Broth (BHI)

Brain Heart Infusion (BHI) broth is a general-purpose liquid medium for the cultivation and maintenance of a wide range of fastidious and non-fastidious microorganisms, including aerobic and anaerobic bacteria, yeast, and molds from a variety of clinical and non-clinical specimens. Preparation of the media is as follows;

- 37 grams of the dehydrated medium was dissolved in 1000 ml of distilled water based on manufacturer's instructions in a conical flask and was mixed thoroughly.
- The conical flask is then closed using a foil cork (made up of cotton wool wrapped in aluminum foil).
- The mixture was stirred for a while using the magnetic stirrer to completely dissolve the powder.
- 5ml of the media was then dispensed into various test tubes and covered with foil cork and was then sterilized by autoclaving at 121˚C for 15minutes.

3.5 Isolation of *Salmonella* and *Shigella* Sp.

3.5.1 Primary Enrichment

Twenty-five (25) grams of each game meat were placed in a sterile stomacher bag containing 225 milliliters of 1% buffered peptone water (enrichment broth) and homogenized for two minutes at 180 revolutions per minute using the stomacher. After homogenization, the material was tra6sferred to conical flasks and incubated at 37˚C for 24hours (Najwa *et al.,* 2015).

3.5.2 Secondary Enrichment

One (1) mls of the Incubated Primary Enrichment was aspirated and dispensed into nine (9) mls of Rapparport-Vassiliadis Soya Peptone Broth in test tubes and incubated at 42˚C for 24 hours. After incubation the tubes were checked for turbidity and turbid tubes were subjected for streaking on Xylose Lysine Deoxycholate Agar.

3.5.3 Plating Method

A loopful of turbid Rapparport-Vassiliadis Soya Peptone Broth tubes was streaked on under aseptic conditions Xylose Lysine Deoxycholate Agar and incubated at 37ºC for 24 hours.

3.5.4 Pure Culture Technique

The plates were observed for growth after the required duration to know if there is a need for sub-

culturing. Sub-culturing was done to purify the isolated bacterial colonies from a mixture culture to a new and single culture, the bacterial isolates sub-cultured were those differentiated based on their colony morphology, shape, colour, elevation and other physical characteristics. Colonies differentiated by morphological characteristics onto fresh petri dishes containing solidified Hektoen Enteric Agar (HEA). A loopful of preferred isolates were picked from the XLD plates using the inoculating loop and streaked on the sterile HEA plates and was incubated at 37ºC for 24 hours.

3.5.5 Cryopreservation of Isolates

A loopful of pure cultured presumptive *Salmonella* and *Shigella* sp. from the incubated Hektoen Enteric Agar (HEA) was inoculated into 5 ml of BHI broth and incubated at 37ºC for 18-24 hours a sterile. After incubating, 750µl of the inoculum was added into a sterile eppendorf tube containing 750µl of sterile 20% sterile glycerol (duplicated) which serves as cryoprotectant and it was stored in a -40C freezer.

3.6 Biochemical Test

3.6.1 Grams Staining

The inoculating loop was sterilized with a bunsen burner flame, and then a pure culture was smeared on a sterile slide and heat fixed by passing it fast across the flame with the smear facing up. For staining, the slides were placed on the staining rack. The smear was covered in crystal violet stain and left for 1 minute before being carefully wiped off under running tap water. The smear was then flooded with Gram's iodine, which was allowed to sit for 1 minute before being drained off under a gentle running tap. The slide was then washed with a decolorizing chemical (70 percent alcohol) and let to stand for 10 seconds. After that, the slide was cleaned under running tap water, drained fully, and counterstained for 30 minutes with safranin. The slide was then blot dried with filter paper after being washed under gently running tap water until no color appeared in the effluent, The slide was then observed under the microscope. (Olutiola *et al.,* 2000).

3.6.2 Catalase Test

Using a sterilized inoculating loop, the pure culture was smeared on a sterile slide. The smear was then treated with a drop of hydrogen peroxide. The outcome was then examined. The existence of

oxygen bubbles indicated that catalase was present, while the absence of bubbles indicated that catalase was absent (Olutiola *et al.,* 2000).

3.6.3 Oxidase Test

The pure culture was smeared on the filter paper, along with a few drops of the oxidase reagent, and the results were examined. Oxidase positive cultures produced a purple tint in less than 10 seconds. Oxidase negative cultures did not develop any purple colour (Olutiola *et al.,* 2000).

3.6.4 Motility Test

The Motility test as the name implies, was carried out to determine if the suspected *Salmonella* and *Shigella* sp. Isolates were capable of moving independently using metabolic energy. Sulphide Indole motility medium was used for motility test. Sulphide Indole Motility medium is a bacterial growth medium that tests for sulfate reduction, indole production, and motility. Preparation is as follows;

- 30 grams of medium was suspended in 1000 ml of distilled water
- The mixture was then stirred for a while using the magnetic rod stirrer hot plate to dissolve the powder completely.
- It was dispensed into tubes and sterilized by autoclaving at 121°C for 15 minutes.

TEST PROCEDURE

- A straight needle was used to touch a colony of a young (18- to 24-hour) culture growing on agar medium.
- The needle was stabbed once to a depth of only $^{1}/_{3}$ to ½ inch in the middle of the tube. The needle was kept in the same line it entered as it was removed from the medium.
- The tubes were incubated at 35°-37°C and examine daily for up to 7 days.
- The tubes were observed for a diffuse zone of growth flaring out from the line of inoculation.

3.6.5 Triple Sugar Iron Test

The Triple Sugar Iron (TSI) test is a microbiological test that evaluates a microorganism's ability to ferment sugars and produce hydrogen sulfide. Triple Sugar Iron Agar was used for this test. Preparation of the agar is as follows;

- 64.42 grams of the dehydrated medium was suspended in 1000 ml purified distilled water

- It was heated to boiling to dissolve the medium completely.
- It was mixed well and distributed into test tubes and sterilized by maintaining at 10lbs pressure (115°C) for 30 minutes.
- The medium was allowed to set in the sloped form with a butt about 2.5cm long.

PROCEDURE

- The top of an isolated colony was touched with an inoculation needle.
- TSI was inoculated by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant.
- The cap was left on loosely and the tube was incubated at 35°-37°C in ambient air for 18 to 24 hours.
- The reaction of medium was examined.

3.7 Molecular Identification

3.7.1 Activation of Isolates

Isolates were taken out of the freezer and allowed to thaw at room temperature. 1ml of BHI was added to Eppendorf tubes and autoclaved. 100µl of *Salmonella* and *Shigella* sp. isolates were added to the Eppendorf tubes containing the BHI and incubated at 37˚C for 24h bringing about the activation of the isolates.

3.7.2 DNA Extraction

The isolates were centrifuged for 5 minutes at 10,000RPM, then the supernatant was decanted. The Eppendorf tube was filled with 1ml of sterile distilled water, vortexed, and centrifuged at 10,000 RPM for 5 minutes, the supernatant was discarded, and the process was repeated.
After which, 200 µl of sterile nuclease-free water was pipetted into the Eppendorf tube containing the pellets, vortexed, and then placed in the heating block to boil at 100˚C for 15 minutes, the solution was then placed in ice to cool, and the contents of the Eppendorf tube were centrifuged for 5 minutes at 14,000RPM. A new set of Eppendorf tubes were labelled with the corresponding codes of the isolates and supernatant containing the extracted DNA was then transferred into the new Eppendorf tubes and stored in the freezer at -20˚C for further analysis.

3.7.3 Polymerase Chain Reaction (PCR)

The components of the PCR used to identify *Salmonella* and *Shigella* sp. are listed in table below. The PCR cocktail was made and then transferred to a PCR tube before being placed in the thermocycler. Initial denaturation was performed at 95°C for 5 minutes, followed by 35 cycles of 95°C for 2 minutes, 42°C for 30 s, and 72°C for 4 minutes, followed by a final elongation step at 72°C for 10 minutes. There were also negative control reactions provided. The template DNA was replaced with sterile water for the negative controls. Electrophoresis was used to confirm the PCR products, and a Gel Documentation system was used to visualize them under UV light.

Table 3.2 Protocol for thermal cycler

Analysis	Step	Temperature	Time
1x	Initial denaturation	95°c	5 min
35x	Denaturation	95°c	2 min
	Annealing	55°c	30 sec
	Elongation	72°c	4 min
1x	Final Elongation	72°c	10 min

Table 3.3: Forward and Reverse primers used for *Salmonella* subspecies 1gene amplification

Primer	Target gene	Target	PCR product size (bp)	Sequences	Reference
STM4057-f	STM4057	*Salmonella* subspecies I	137	5' -GGTGG CCTCG ATGAT TCCCG-3'	(Kim *et al.,* 2006a)
STM4057-r				5' -CCCAC TTGTA GCGAG CGCCG-3'	(Kim *et al.,* 2006a)

Table 3.4: Components of PCR for *Salmonella* subspecies 1

Reagent	Initial concentration	Final concentration	Volume/Reaction
Master Mix	5x	1x	2 µl
Forward Primer	20µm	0.4µm	0.2µl
Reverse Primer	20µm	0.4 µm	0.2µl
dH_2O			5.6µl
DNA			2 µl
Total			10

Table 3.5 PCR reaction components used for 16s rRNA amplification

Component	Initial concentration	Final concentration	Volume/rxn
Master mix	5x	1x	2ul
16sf	20um	0.25um	0.125ul
16sr	20um	0.25um	0.125ul
DNA			2ul
dH_2O			5.75ul
Total			10ul

3.7.4 Agarose Gel Electrophoresis

Dry Agarose powder was used to make the agarose, and 1.8g of it was dissolved in 100ml of 1x TAE buffer. The mixture was heated until a clear solution was obtained. A micropipette was used to add 3µl of ethidium bromide to the mixture. It was then spun, allowed to cool and then transferred to the gel tray which results in a slab with the combs in place, that create wells in the slab. It was then allowed to solidify before carefully removing the combs. The gel tray containing the slab was placed into the gel tank and 1x TAE buffer was added to the gel tank.

3 µl of DNA ladder was added to the first well, and 4 µl of amplicon (one sample per well) was pipetted into each well that was produced. The tank was attached to the power pack and the gel was ran at 100 volts for 45 minutes. The resulting gel was inspected using the gel documentation system.

3.8 Precautions

Personal protective equipment, such as a covered shoe, a nasal cover, gloves, and a lab coat, were observed. At every stage of the project, aseptic practices were observed. There was no cross-contamination of the samples. Ensured that the samples were appropriately labeled at all times. To avoid killing the organism of interest, the inoculating loop was allowed to cool before making contact with the organism for sub-culturing.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

Microbial analyses of 55 game meat sampled which consist of 15 Antelopes, 7 Grasscutters, 6 Rabbits, 4 Civet cats, 4 Guinea Fowls, 3 Deers, 2 Alligators, 2 Pangolins, 2 Hedgehogs, 2 Sparrow, 3 Bushdogs, 2 Monkeys, 1 Hare, 1 Porcupine, 1 Quail, was performed for detection of *Salmonella* and *Shigella* species. The morphological characteristics of the isolates were critically observed so as to predict the possible presence of suspected *Salmonella* and *Shigella* species. A total of 75 *Salmonella* isolates and 57 *Shigella* isolates were recovered.

4.2 Biochemical Test of *Salmonella* sp.

The results of the biochemical tests indicated that all of the *Salmonella* isolates were Gram negative, they were all positive for catalase test and all negative for oxidase test and all were positive for motility test, positive result was also observed in all isolates for triple sugar iron test as shown in Table 4.1

Table 4.1: Biochemical Test results of the presumptive *Salmonella* isolates

Sample	Gram reaction	Catalase	Oxidase	Motility	TSI	Probabale organism
AN_1S_1	-	+	-	M	+	*Salmonella* sp.
RAS_1	-	+	-	M	+	*Salmonella* sp.
IGS_1	-	+	-	M	+	*Salmonella* sp.
RAA_1	-	+	-	M	+	*Salmonella* sp.
GUA_1	-	+	-	M	+	*Salmonella* sp.
CC_1O_1	-	+	-	M	+	*Salmonella* sp.
RAO_1	-	+	-	M	+	*Salmonella* sp.
AN_1O_1	-	+	-	M	+	*Salmonella* sp.
GR_1O_1	-	+	-	M	+	*Salmonella* sp.
PA_2L_1	-	+	-	M	+	*Salmonella* sp.

4.3 Biochemical Test for *Shigella* sp.

The results of the biochemical tests indicated that all of the *Salmonella* isolates were Gram negative, they were all positive for catalase test and all negative for oxidase test and all were negative for motility test, positive result was also observed in all isolates for triple sugar iron test as shown in Table 4.2.

Table 4.2: Biochemical test results of the presumptive *Shigella* isolates

Laboratory Code	Gram reaction	Catalase	Oxidase	Motility	TSI	Probable organism
GRS_1	-	+	-	N	+	*Shigella* sp.
RAS_1	-	+	-	N	+	*Shigella* sp.
BUS_1	-	+	-	N	+	*Shigella* sp.
ANA_1	-	+	-	N	+	*Shigella* sp.
HEA_1	-	+	-	N	+	*Shigella* sp.
GUA_1	-	+	-	N	+	*Shigella* sp.
ALA_1	-	+	-	N	+	*Shigella* sp.
CC_1O_1	-	+	-	N	+	*Shigella* sp.
RAO_1	-	+	-	N	+	*Shigella* sp.
AN_1O_1	-	+	-	N	+	*Shigella* sp.

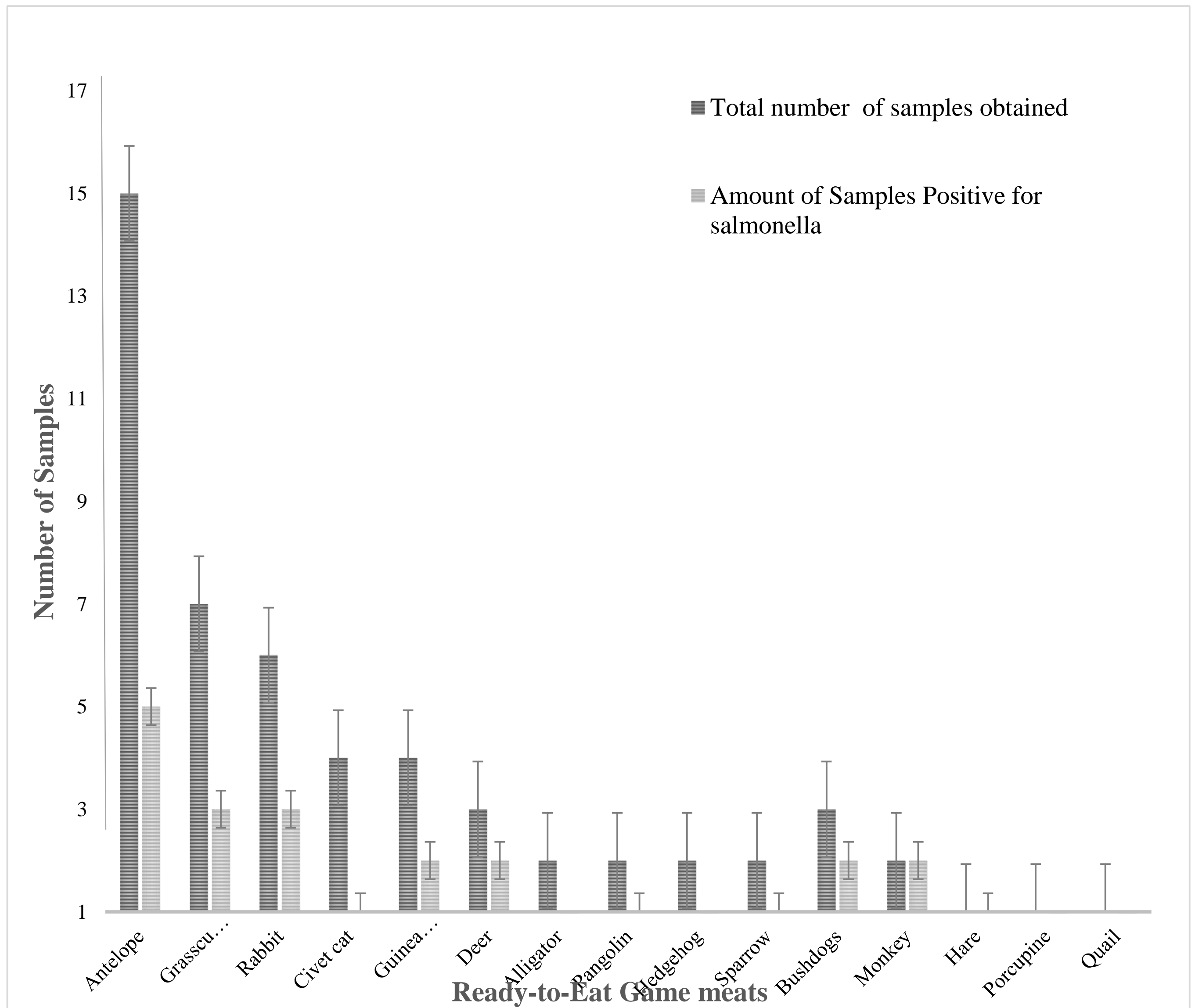

Figure 4.1: Samples positive for *Salmonella* out of the total different game meat samples obtained

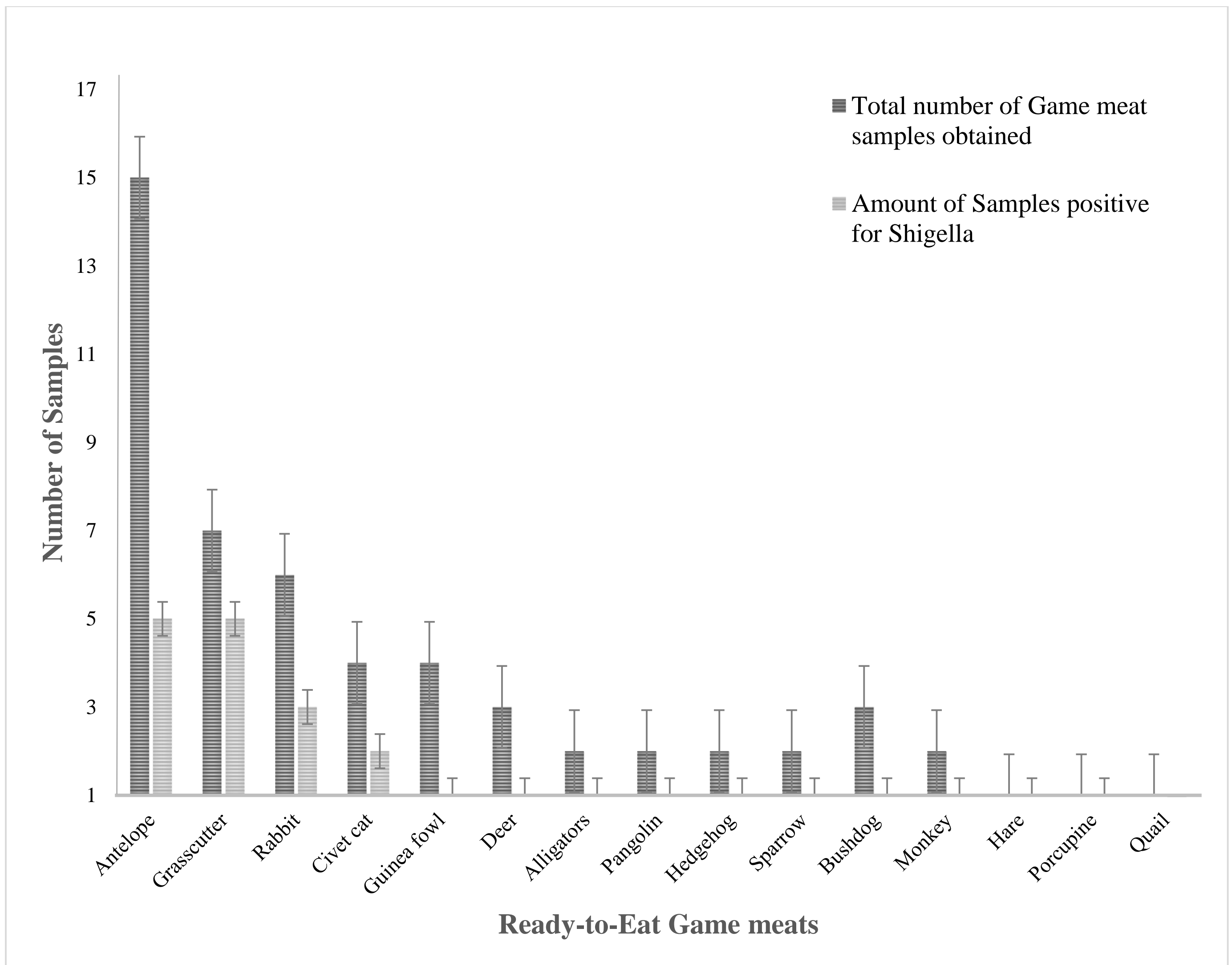

Figure 4.2: Samples positive for *Shigella* out of the total different game meat samples obtained

4.4 Molecular Identification of isolates

50 *Salmonella* isolates and 30 *Shigella* isolates were randomly selected from the 75 and 57 isolates respectively for gene amplification. The *Salmonella* Subspecies I gene of the *Samonella* isolates was amplified and the 16s rRNA gene of the *Shigella* isolates was amplified. The Simplex-PCR products were quantified using agarose gel electrophoresis and image of the gel was generated as shown in Figure 4.3 and 4.4 below.

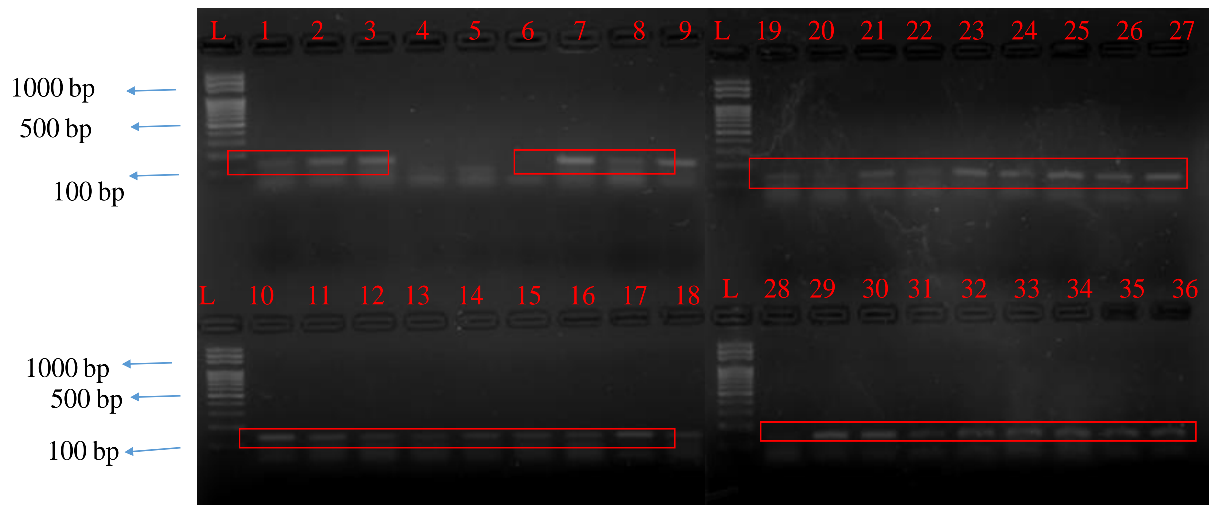

Plate 4.1: Illustrative agarose gel electrophoresis image of a Simplex PCR assay of game meat samples for detecting the *Salmonella enterica* subsp. I (SSI) gene (137 bp). Lane L: molecular weight marker 100 bp (Bio-Mark). Lane 1: positive control strain (*Salmonella Typhimurium* CCM 7205). Lanes 2-36: *Salmonella* sp. strain isolated from game meat

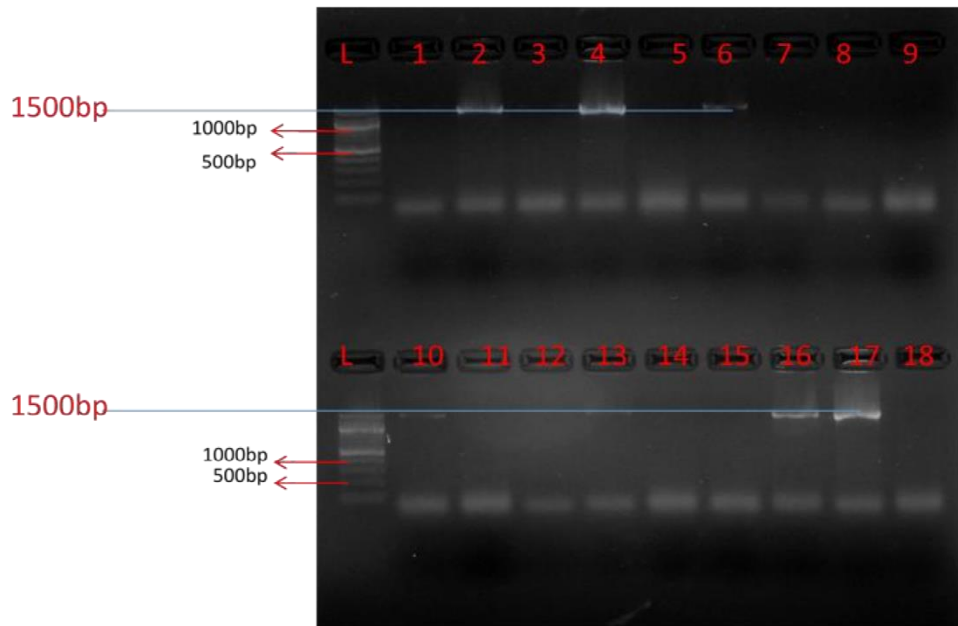

Plate 4.2: Illustrative agarose gel electrophoresis image of PCR amplified products of 16 rRNA strains. Lane L: molecular weight marker 100 bp (Bio-Mark). Lane 1-10: *Shigella* sp. strain isolated from game meat.

Table 4.3: Prevalence of *Salmonella* and *Shigella* sp. in the investigated game meat samples

Game meat Investigated	Number of samples	Number of samples positive for *Salmonella* sp.	Prevalence (%)	Number of samples positive for *Shigella* sp.	Prevalence (%)
Antelope	15	5	33.3	5	33.3
Grasscutter	7	3	42.9	5	71.4
Rabbit	6	3	50	3	50
Civet Cat	4	1	25	2	50
Guinea Fowl	4	2	50	1	25
Bushdog	3	2	66.7	1	33.3
Deer	3	2	66.7	1	33.3
Alligator	2	0	0	1	50
Pangolin	2	1	50	1	50
Hedgehog	2	0	0	1	50
Sparrow	2	1	50	1	50
Monkey	2	2	100	1	50
Hare	1	1	100	1	100
Porcupine	1	0	0	1	100
Quail	1	0	0	0	0
Total	55	23	41.8	25	45.5

4.5 Discussion

The worldwide burden of sickness and mortality from salmonellosis and shigellosis remains high (Rahman *et al.,* 2012). Due to the food handlers' poor health, as well as their lack of personal hygiene, knowledge, and experience, the transmission primarily happens through the food chain (from fingers to food, and then to customers) (Mengist *et al.,* 2018). The spread of foodborne infections is further facilitated by ineffective food safety laws, poor food handling and sanitation standards, and weak regulatory frameworks (Scallan *et al.,* 2011).

In this study the prevalence of *Salmonella* and *Shigella* sp. in the game meats samples investigated were 41.8% and 45.5% respectively. Out of the total 55 game meats samples that were analyzed, 23 and 25 were positive for *Salmonella* and *Shigella* sp. respectively. This high prevalence of *Salmonella* and *Shigella* sp. in this current study supported previous reports on high levels of these pathogens in game animals and ready-to-eat-game meats (Gill, 2007; Rhoades *et al.,* 2009; Bachand *et al.,* 2012; Guerra *et al.,* 2016).

According to the amended EU Regulation 2073/2005, it is required that *Salmonella* and *Shigella* must be absent in 25g of food sample (Ehuwa *et al.,* 2021; Cortes-Sanchez, *et al.,* 2021). The reason for the high prevalence of *Salmonella* and *Shigella* sp. in the investigated game meat samples could be due to the conditions under which the animal is killed, as well as how the carcass is prepared and slaughtered (Soepranianondo *et al.,* 2019; Paulsen *et al.,* 2012). Also the majority of game animals from which meat is harvested are slaughtered on the field. The animal may be surrounded, trapped, or brought to the ground by hunting dogs before being killed with a knife or other bladed weapon. But nowadays, the majority of huge game would be taken down with a rifle bullet or, much less frequently, an arrow or a bolt from a bow, all these are probable sources of biological contamination of the game meat (Gill, 2007).

Furthermore, during the course of sampling for this study, it was observed that the commercial ready to eat game meat producers have little or no food safety measures in place, some of the ready-to-eat-game meat vendors actually used their bare hands which might have been previously contaminated to sell meat to the consumers. The high prevalence could also be attributed to the unhygienic practices during processing of the carcass, during the course of sampling it was observed that slaughtering of the animals, evisceration and splitting of carcass is usually carried out on bare floor and poor quality water is also used for washing carcasses. These highlighted

observations in addition to high ambient temperature, humidity and poor handling practices can dispose the meat to deterioration and contamination during transportation, processing and sale (Uçar, 2016). Also, the unhygienic condition of the surfaces on which the processed game meat were displayed for sale in the open markets could also contribute to contamination (Salihu *et al.,* 2013).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions and Recommendations

The findings of this study established high prevalence rate of *Salmonella* and *Shigella* sp. in Ready-to-Eat game meat sold at open markets in different cities of southwestern Nigeria. The presence of these two pathogens indicates that the hygiene of ready-to-eat game meat is compromised in these cities, this is a situation that calls for public health concern. All of the findings and discussions from this study support the need to pay even more attention to how game meat is prepared and to take effective safety measures to protect human health. It is necessary to implement an effective food safety management system that will ensure a significant reduction in contamination of meats and ready-to-eat meat products with foodborne pathogens. There is a need to educate the general people about the dangers of consuming contaminated game foods. The trade in game meat should also be regulated by appropriate regulatory bodies. Surveillance methods should be put in place to adequately track and monitor the occurrence of foodborne infections caused by the consumption of game meat. Sanitation and hygienic conditions in the preparation and selling of ready-to-eat game meat should be closely monitored, particularly in the open market, in order to reduce or minimize the occurrence of foodborne pathogens.

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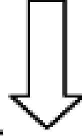
APPENDICES

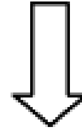
Non-selective enrichment

25 g samples will be added in a stomacher bag containing
225 ml of buffered peptone water and incubated at 37℃ for 24 h



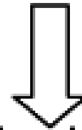
Selective enrichment

0.1 ml in 10 ml Rappaport-Vassiliadis Soy Broth and incubate at 37℃ for 24 h



Isolation

Xylose-Lysine-Desoxycholate (XLD) and incubate at 37℃ for 24 h



Biochemical tests

Indole, Simmon citrate, Urease and incubate at 37℃ for 24 h

Appendix 1: Flow diagram showing ISO 6579: 2002method for detection of *Salmonella* and *Shigella*.

Appendix 2: Composition and preparation of culture media and reagents

A) Buffered peptone water (BIOMARK)

Composition (g/Litre):

Proteose peptone	10.0 g
Sodium chloride	5.0 g
Disodium phosphate	3.5.0 g
Monopotassium phosphate	1.50 g
Water	1 000 ml

Final pH (at 25°C): 7.2 ± 0.2

Preparation: Add 20 gram of the components in the 1000 ml of distilled water, Mix well and distribute into universal bottle of suitable capacity to obtain the portions necessary for the test. Sterilize by autoclaving for 15 min in the autoclave set at 121 °C.

B) Rappaport -Vassiliadis (RV) Soya enrichment broth of 500 g (Merck KGaA,Germany)

Composition (g/Litre):

Enzymatic digest of Soya…………...4.5 g

Soya peptone ……………….……….5.0 g

Sodium chloride……………………..7.2 g

Potassium dihydrogen phosphate ….1.26 g

Magnesium chloride anhydrous …...13.4 g

Malachite green…………………...0.036 g

Preparation: Weigh 30 g (the equivalent weight of dehydrated medium per Litre) and add to 1 Litre of distilled water. Heat gently until completely dissolved. Dispense 10 ml volumes into screw capped bottles or tubes and sterilize by autoclaving at 115℃ for 15 minutes.

C) Xylose lysine deoxycholate agar (XLD agar) 500 g (Merck KGaA,Germany)

Composition (g/Litre):

Yeast extract………………………….. 3.0

Lactose monohydrate………………….7.5

D (+)- Xylose…………………………3.5

L(+)- Lysine…………………………..5.0

Sucrose…………………………………7.5

Sodium deoxycholate…………………..2.5

Sodium chloride………………………..5.0

Sodium thiosulphate……………………6.8

Iron (III) ammonium citrate……………0.8

Phenol red……………………………...0.08

Agar-agar..……………………………..13.5

pH 7.4 ± 0.2 at 25℃

Preparation: Suspend 55.2gm in 1000 (1 Litre) of distilled water by heating, with frequent agitation, until the medium starts to boil. Avoid overheating. Adjust the pH, if necessary, so that after sterilization it is 7.4 at 25 °C. Heat with frequent agitation until the medium boils and the agar dissolves. Do not overheat. Transfer immediately to a water bath at 50 °C, agitate and pour into plates. Allow to solidify. Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven set between 37 °C and 55 °C until the surface of the agar is dry. It is advisable not to prepare large volumes which will require prolonged heating.