ISOLATION AND MOLECULAR CHARACTERIZATION OF SHIGA TOXIN PRODUCING *Escherichia coli* (STEC) ASSOCIATED WITH READY-TO-EAT GAME MEAT IN SOUTH WEST, NIGERIA

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DECLARATION

I hereby declare that this project written under the supervision of Dr. O. E. Fayemi is a product of my own laboratory analysis and literature search. Information derived from various sources have been duly acknowledged in the text and a list of references provided. This Project research has not been presented anywhere for the award of any degree or certificate.

CERTIFICATION

This is to certify that this Project research titled "**ISOLATION AND MOLECULAR CHARACTERIZATION OF SHIGA TOXIN PRODUCING *Escherichia coli* (STEC) ASSOCIATED WITH READY-TO-EAT GAME MEAT IN SOUTH WEST, NIGERIA**" was carried out by **ADEYA, CHIDINMA CELINE**, with matriculation number **18010101026**. This Project meets the requirements governing the award of the **Bachelor of Science (B. Sc.)** degree in Microbiology department of biological sciences of Mountain Top University, Ogun State, Nigeria and is approved for its contribution to knowledge.

_____ _____

Dr. O. E. FAYEMI — Date

(Project Supervisor)

_____ _____

Dr. (Mrs.) C. I. AYOLABI — Date

(Ag. Head of Department)

DEDICATION

My utmost gratitude goes to God for making this research, and my stay throughout school a success, for unending divine provision, insight and help, I am forever grateful.

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I dedicate this work to the Almighty God for his grace, health and everything needed to make this work a success.

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ABBREVIATIONS

BHI – Brain Heart Infusion broth

BPW – Buffered Peptone Water

CFU/ml – Colony Forming Units/ml

DAEC – Diffusely Adherent *Escherichia coli*

DEC – Diarrheagenic *Escherichia coli*

DPEC – Diarrheagenic Pathogenic *Escherichia coli*

ExPEC – Extra-intestinal Pathogenic *Escherichia coli*

EAggEC – Enteroaggregative *Escherichia coli*

EHEC – Enterohemorrhagic *Escherichia coli*

EIEC – Enteroinvasive *Escherichia coli*

EPEC – Enteropathogenic *Escherichia coli*

ETEC – Enterotoxigenic *Escherichia coli*

HUS – Hemolytic Uremic Syndrome

MAC – Mac Conkey agar

NA – Nutrient agar

NMEC – Neonatal Meningitis *Escherichia coli*

PCR- Polymerase Chain Reaction

SMAC – Sorbitol Mac Conkey agar

STEC – Shiga toxin producing *Escherichia coli*

TVC – Total Viable Count

UPEC – Uropathogenic *Escherichia coli*

WHO -World Health Organization

ABSTRACT

Game meat is a term that includes all animals derived from wildlife. It is indigenous to China and Africa. In Nigeria, a variety of game meat is consumed, Grasscutter and Antelope being the dominant species consumed. The presence of pathogenic microorganisms in the game meat that is commonly consumed may spell doom to the public health, and conversely the society at large. Therefore, this research was carried out to determine the presence of pathogenic *Escherichia coli*, specifically Shiga toxin producing *E. coli* in various game meat species sourced from Lagos, Ogun, Oyo, Ondo and Osun State in the South West region of Nigeria. A total of 55 game meat samples were aseptically obtained at designated sales points and *E. coli* was isolated using Sorbitol Mac Conkey agar (SMAC) and Mac Conkey agar (MAC). Morphological and biochemical tests were performed on the isolates. The suspected isolates were then genotypically characterized using Multiplex PCR. Among the game meat sampled (n=55), 25.4% of the game meat samples were confirmed positive for Shiga toxin producing *E. coli* genes, out of which 12.7% and 3.6% possessed the Enterotoxigenic Human and Porcine estA gene 9.1%, positive with the *Vtx1* gene that produces Shiga toxin. This means that few of the game meat samples were Shiga Toxin producing *E. coli* (STEC). The presence of pathogenic *E. coli* is a risk to the public health as it could cause foodborne illnesses and diseases including diarrhea and Hemolytic Uremic Syndrome (HUS).

Keywords: *Escherichia coli*, Game meat, PCR, Shiga toxin producing *Escherichia coli*, South west.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Game meat refers to any animal gotten from wildlife. Game meat is used as a source of food for man, and in recent times, as a profitable source of income. In global distribution, game meat is indigenous to Africa and Asia (Ariyo *et al*., 2018). Game meat is most commonly referred to as 'bush meat' in Nigeria (Okeke *et al*., 2013). The consumption of game meat is on the rise, and this might pose a threat to the general public health as Game meat is said to be a zoonotic agent (Gomes-Neves *et al*., 2021). The presence of pathogenic microorganisms in game meat consumed is of great concern as this poses risk to the health of the consumers which may lead to the outbreak of several foodborne diseases (Fayemi *et al*., 2021). The microorganisms that have been associated with game meat includes *Toxoplasma gondii* (Almeria *et al*., 2021), *Trichinella nativa* (McIntyre *et al*., 2007), Enterohemorrhagic *Escherichia coli*, *Clostridium botulinum, Clostridium perfringes, Campylobacter spp, Salmonella spp, Shigella spp* (Todd, 2014).

Escherichia coli is one of the most important abundant microorganisms found distributed in nature owing to its ubiquitous nature (Kolenda *et al*., 2015). Diarrheagenic pathogenic *Escherichia coli* (DPEC) strains are among the most important microorganisms known for causing foodborne diseases, and has also been implicated as the most common causative agent of diarrhea (Mohamed *et al*., 2018).

Diarrhea as defined by the World health Organization (WHO), is the excessive, frequent passing of watery stools for a duration of 3 -7 days. Diarrheal diseases however preventable and treatable, is the second leading cause of death in children under 5 years globally, with an annual mortality rate of 520, 000 [World Health Organization, 2017]. In Africa, diarrheal diseases are ranked among the main causes of morbidity and mortality affecting children under 5 (Adjuik *et al*, 2006). The Shiga toxin Producing *Escherichia coli* (STEC), a diarrheagenic pathotype is known to cause diarrhea and serious potentially life-threatening diseases such as the Hemolytic Uremic Syndrome (Ayoade *et al*., 2021).

1.2 STATEMENT OF PROBLEM

The ready-to-eat game meat sold by different vendors from various locations in Nigeria are not always prepared under standard conditions, leaving a high probability of being contaminated with pathogenic microorganisms. This is a call for concern on the food safety in the consumption of these game meat as it could lead to an unpredictable outbreak of food borne diseases such as diarrhea or in worst case even zoonotic endemic or epidemic diseases as game meat is a notable source of zoonotic diseases.

1.3 JUSTIFICATION OF STUDY

The consumption of game meat in Nigeria is experiencing an increase. The presence of pathogenic *E. coli*, particularly the Pathotype Shiga Toxin Producing *Escherichia coli* (STEC) will be investigated in the Game meat to ascertain the safety of consuming game meat. Therefore, this study is necessary for the investigation of the microbiological quality and safety in consuming game meat.

1.4AIM AND OBJECTIVES

The aim and objectives of this study are as follows.

- To isolate *Escherichia coli* in ready-to-eat game meat from various locations within South western Nigeria.
- To determine the virulence genes present in the isolated Shiga Toxin Producing *Escherichia coli* (STEC).

CHAPTER TWO

LITERATURE REVIEW

2.1 GAME MEAT

Game meat refers to any animal gotten or sourced from wildlife. Game meat was formerly the main source of meat that was available for consumption before the domestication of some species (Olaniyan *et al*., 2016). In Past times it was a food source for personal consumption by people indigenous to traditional settings such as villages. Recently, this is not the case, game meat consumption is becoming more accepted as it is not only limited to the hunters and traditional indigenes in remote areas but now more appreciated due to its nutritional value (Gomes-Neves *et al*., 2021). The consumer interest of game meat is on the rise due to the rapidly growing population of wild game species (Branciari *et al*., 2022). Game meat is high in Protein, mineral content and low fat and cholesterol levels (Lizana *et al*., 2022). A quantitative based survey showed that people consume game meat based on rational motives such as preference of taste, nutritional value and availability (Niewiadomska *et al*., 2020). Commercial hunting has allowed and encouraged the sales of different species of bush meat at pricey rates in specific cities, towns known as 'hotspots', which does not only provide the availability of bush meat for consumption but also serve as a lucrative source of income (Okeke *et al*., 2013). In Africa, Antelope is the most prevalent game meat consumed (Faraz and Waheed, 2018).

2.2 IMPLICATION OF GAME MEAT IN FOOD BORNE DISEASES

Given that there are no hygienic standards or norms in the wild, the origin of game meat is a major cause for concern. Due to the hunting procedure, the hygiene throughout the post-hunting process, processing, and transportation, the safety of game meat is frequently questioned (Gomes-Neves *et al*., 2021). To reduce the potential risk to the consumer, it is crucial to ensure the sanitation and safety standards of game meat. It has been shown that 43% of the foodborne pathogens

linked to recent outbreaks of infectious diseases around the world are descended from wildlife species (Hedman *et al*., 2020). Shiga Toxin producing *Escherichia coli* is a zoonotic agent associated with game meat and its products, which could compromise the safety, hence, leading to the development of foodborne diseases such as diarrhea, hemorrhagic colitis, etc. (Naseer *et al*., 2017).

According to reports, game meat sold in the local market in Wuhan, China, was a source of the SARS-CoV-2 virus outbreak, which was recognized as the first respiratory foodborne illnesses to spread globally (Jalava, 2020). A further important factor in ensuring the safety of consuming game meat is the hygiene involved, which relates to the processes, protocols, and procedures for promoting health.

2.3 *ESCHERICHIA COLI*

2.3.1 BRIEF HISTORY OF *ESCHERICHIA COLI*

Escherichia coli was first discovered and described by the German pedriatrist and microbiologist Theodor Escherich. In 1884, Theodor Escherich focused his research on the bacterial flora of infantile diarrhea (Kaper *et al*., 2005). Theodor described *Escherichia coli* as normal inhabitants of the intestine and also proposed that they were responsible for some infectious diseases of the intestine and the urinary tract (Friedmann, 2014). He isolated 19 different bacteria from the stool samples of children he observed, carried out Gram staining on the organisms and described in detail the organism *Bacterium coli* commune, now known as *Escherichia coli*. (Shulman *et al*., 2017).

2.3.2 CHARACTERISTICS OF *ESCHERICHIA COLI*

Escherichia coli is a Gram-negative bacilli of length 1µm and width of 0.35µm (Malik *et al*., 2022). *Escherichia coli* are non-sporulating Facultative anaerobes and is a member of the Enterobacteriaceae family, hence, constitutes a part of the normal micro flora of the gut (Saba *et al*., 2015). *E. coli* is ubiquitous in nature; as it is found in soil, water, food due to contamination from faeces or from animals (Kolenda*et al*., 2015). *E. coli* becomes pathogenic as a result of acquisition of virulent genes by mobile elements such as transposons, plasmids bacteriophages and even through

pathogenicity islands (Campilongo *et al*., 2014; Razzaq *et al*., 2016). Certain food products like meat, dairy products, vegetables have been implicated in food borne disease outbreak caused by *Escherichia coli.*(Saad *et al*., 2021). It is regarded as the most studied bacterial specie because it can easily be propagated and easy to be genetically manipulated (Abdelrahman *et al*., 2022).

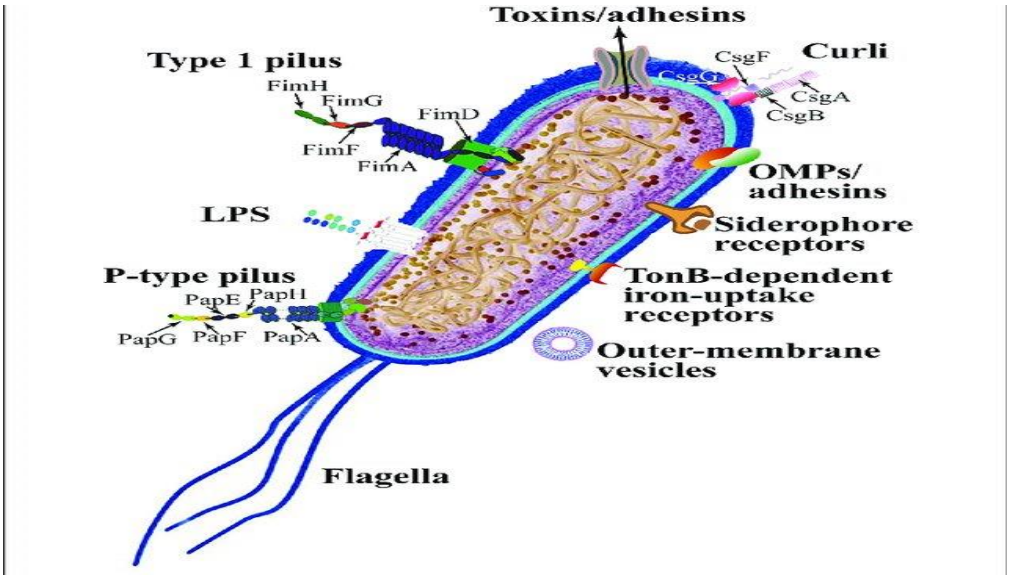


Figure 2.1: Detailed Structure of *Escherichia coli* Adapted from Terlizzi *et al* (2017).

2.3.3 PATHOTYPES OF *ESCHERICHIA COLI*

Several *Escherichia coli* strains have been discovered and have been grouped as either Extra-intestinal pathogenic *Escherichia coli* (ExPEC) or Intestinal pathogenic *Escherichia coli*. The intestinal pathogenic *Escherichia coli* otherwiseknown as Diarrheagenic *Escherichia coli* (DEC) comprises of 6 pathotypes, they include Enterotoxigenic *Escherichia coli* (ETEC), Enteropathogenic *Escherichia coli* (EPEC), Diffusely Adherent *Escherichia coli* (DAEC), Enteroinvasive *Escherichia coli* (EIEC), Enteroaggregative *Escherichia coli* (EAggEC) and the Shiga toxin producing *Escherichia coli* (STEC), (Sikorski *et al*., 2021). Among the Extra-intestinal pathogenic *E. coli*, there are two major pathotypes; Uropathogenic *Escherichia coli* (UPEC) and Neonatal Meningitis *Escherichia coli* (NMEC).

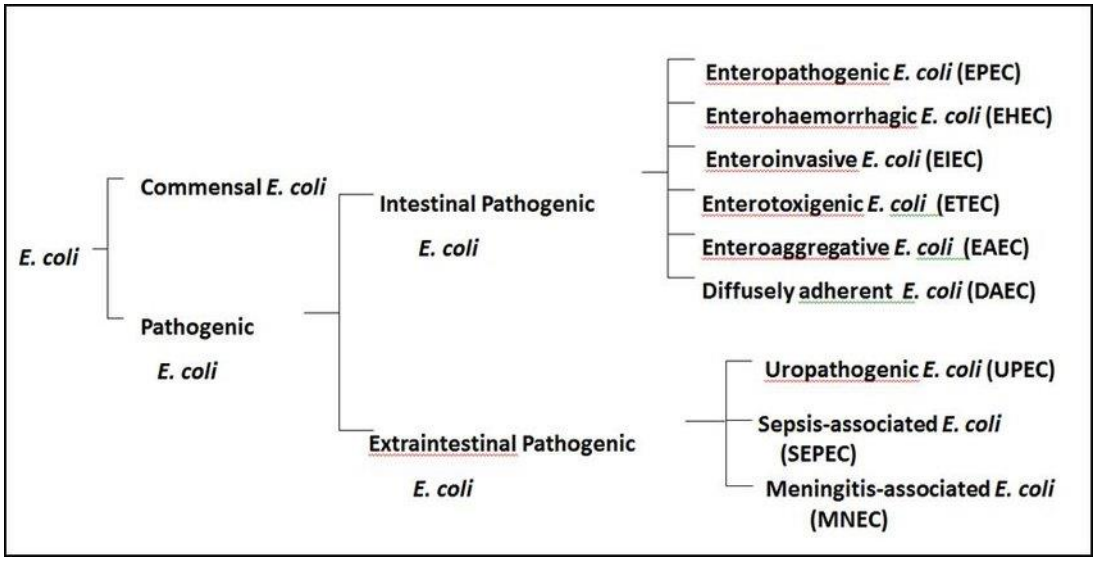


Figure 2.2: Classification of *Escherichia coli* pathotypes Adapted from Wakeham (2013).

2.3.3.1 ENTEROTOXIGENIC *ESCHERICHIA COLI* (ETEC)

Enterotoxigenic *Escherichia coli* is the leading cause of diarrhea in developing countries. As the name implies, Enterotoxigenic *E. coli* possess two enterotoxins used for causing diarrhea namely; heat labile and heat stable enterotoxins respectively (Gomez-Aldapa *et al*., 2013). The major virulence factor possessed by ETEC is the fimbriae, that facilitate attachment to the lumen, followed by the secretion of enterotoxins to induce fluid efflux from the intestine (Curtis *et al*., 2017).

2.3.3.2 ENTEROPATHOGENIC *ESCHERICHIA COLI* (EPEC)

Enteropathogenic *Escherichia coli* (EPEC) is the most implicated organism in infantile diarrhea (Al-Charrakh *et al*., 2012). The mechanism of pathogenicity involves the localized adhesion to the intestinal mucosa and effacement (tight binding) to the intestinal mucosa.

2.3.3.3 ENTEROAGGREGATIVE *ESCHERICHIA COLI* (EAggEC)

The Enteroaggregative *Escherichia coli* (EAggEC), is an *E. coli* pathotype that has been linked and associated with acute and persistent diarrhea in children, and traveler's diarrhea (Mahindroo *et al*., 2021). The mechanism of causing diarrhea is said to be a complex process, however, detailed research has proven that there are 3 necessary EAggEC to be implicated as the causative organism of diarrhea (Mavarro-Garcia and Elias, 2011). The first stage, is the colonization of the intestinal mucosa by the abundant adherence of aggregates of *E. coli* to the intestinal mucosa. The second stage involves the release of enterotoxins and cytotoxins to the colonized intestinal mucosa. The final stage is for the bacteria to induce inflammation of the intestinal mucosa, hence, stimulating diarrhea [Mavarro-Garcia and Elias, 2011]. This pathotype is characterized by the clinical manifestation of watery diarrhea (Savavino *et al*., 1996).

2.3.3.4 DIFFUSELY ADHERENT *ESCHERICHIA COLI* (DAEC)

Diffusely adherent pathotype of *E. coli* is characterized by the presence of a diffusely adherence pattern on HeLa and HEp-2 epithelial cells and associated with Persistent and acute diarrhea in children (Javadi *et al*., 2020). The Diffusely Adherent *E. coli* is detected by distinguishable diffusely adherence to the tissue of the intestinal lining without possessing Hemagglutinin (Yamamoto *et al*., 1996). About 75% of Diffusely Adherent *E. coli* possess adhesins from the Afa/Dr family responsible for the presentation of adhesion (Mansan-Almeida *et al*., 2013)

2.3.3.5 ENTEROINVASIVE *ESCHERICHIA COLI* (EIEC)

The Enteroinvasive *Escherichia coli* (EIEC) was first found in 1971, and was implicated for causing diarrhea in supposedly healthy people (Abbasi *et al*., 2015). Enteroinvasive *E. coli* possess a mechanism of pathogenicity resembling *Shigella dystenteriae* (Pasqua *et al*., 2017). As the name implies, Enteroinvasive *E. coli* invades the lining of the epithelium and facilitates spreading of the bacteria to colonize the surface with its enterocytes. The serotype O96:H19 is capable of also producing biofilms to colonize the epithelium of the intestine (Gomez-Duarte *et al*, 2018).

2.3.3.6 SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* (STEC)

As the name implies, Shiga toxin producing *E. coli* is a pathotype of *E. coli* that produces Shiga toxin. This Shiga toxin produced is similar to the toxin produced by the bacteria *Shigella dysenteriae*. It is otherwise known as Enterohaemorrhagic *Escherichia coli* (EHEC) or Verotoxigenic *E. coli* Shiga toxin Producing *E. coli* is a foodborne zoonotic agent that has been associated with global outbreaks that was responsible for serious public health implications (Nguyen & Sperandio, 2012). Shiga toxin Producing *Escherichia coli* (STEC) results in several foodborne illnesses from mild diarrhea to severe cases like Hemolytic Uremic Syndrome (HUS) which damages the kidney (Ayoade *et al*., 2021). The genesis of most infections caused by STEC are due to the common serotype O157:H7, which has been linked to the consumption of contaminated food and water containing the faeces of infected animals (Saba *et al*., 2015). In 1982, the Shiga toxin producing *E. coli* (STEC)

serotype O157:H7 was discovered as a pathogen (Omer *et al*., 2018). STEC is further divided into two serogroups; O157 and non-O157 groups (Babolhavaeji *et al*., 2021). STEC colonizes the intestines and produces Shiga toxins that stimulate inflammatory response, hence, characterized by the clinical manifestation of bloody diarrhea.

2.3.4 EPIDEMIOLOGY OF *ESCHERICHIA COLI*

An estimated 265,000 STEC infections occur in the United States of America yearly ranging from mild to potentially life-threatening infections i.e Hemolytic Uremic Syndrome (Tack *et al*., 2021). Outbreaks of *E. coli* O157:H7 and similar organisms known for producing the Shiga toxin have occurred in many countries including Australia, Japan, the UK, and many other European countries (Todd, 2014; Pennington *et al*., 2014). The epidemiology of diarrheal diseases in Africa caused by the Diarrheagenic *Escherichia coli* (DEC) is rather poorly understood as a result of lack and/or inadequate facilities needed to study and characterize them (Cissé *et al*, 2017). However, countries in Africa which have reported data comprise of Kenya, Nigeria, Côte d'Ivoire, and the Central African Republic, the O157 STEC have been isolated from sporadic cases of diarrhea and HUS, and have also been associated with some diarrheal disease outbreaks, especially in southern Africa. In Nigeria, Non-O157 STEC have also been linked to sporadic cases and outbreaks of diarrhea (Fairbrother & Nadeau, 2006).

2.3.5 PATHOGENESISOF *ESCHERICHIA COLI*

Escherichia coli infections usually develop as a result of ingestion of Contaminated meat or meat product containing *E. coli,* usually STEC (Tack *et al*, 2021).

The consumed pathogenic *Escherichia coli* then replicates within the gut and colonizes the lining of the intestinal mucosa. The *eaeA* gene in STEC is responsible for encoding the protein, intimin, which facilitates the adhesion and strong effacement to the lining of the intestinal mucosa (Khalifa *et al*, 2019). The STEC then secretes either one or both Shiga toxins; Stx1 and Stx2 that corrodes the lining of the intestine, leading to the loss of electrolytes, often presented as watery stool (diarrhea) which may/ may not be bloody (Konate *et al*., 2017).

2.3.6 TRANSMISSION OF ESCHERICHIA COLI

Escherichia coli could be transmitted directly from the consumption of animals such as dogs, cats, livestock; Cattle, sheep, game birds or wild game reservoirs such as wild dog, rabbits, deers, wild cats etc (Lutwick, 2014). *Escherichia coli* could also be transmitted from person-to-person by fecal-oral route (Fairbrother & Nadeau, 2006).

2.3.7 ANTIMICROBIAL RESISTANCE AND SUSCEPTIBILITY OF *ESCHERICHIA COLI*

Resistance to antibiotics is a major problem that has paralleled the discovery of antibiotics. The misuse and abuse of antibiotics at sub-inhibitory concentrations has led to the development of adaptive resistance (Ghosh *et al*., 2019). Evidence from surveillance data shows that resistance in *Escherichia coli* is highest for antibiotics and antimicrobial agents that have been in use for the longest period of time in both human and animal medicine (Issa *et al*., 2020). *E. coli* of animal origin have been reported to often show resistances to relatively older antimicrobial agents, including Tetracyclines, Phenicols, Sulfonamides, Trimethoprim, and Phosphomycin (Poirel*et al*., 2018).In recent times, the increasing development of multi-drug resistance to antibiotics displayed by *E. coli* isolates, including Shiga Toxin Producing *Escherichia coli* is alarming (Davies and Davies, 2010). There is therefore the need for antibiotics susceptibility testing for effective anti-biotherapy of detected STEC isolates (Aguilera-Alonso *et al*., 2022). However, despite being resistant to different classes of antibiotics, STEC is also susceptible to antibiotics when used in the correct concentrations.

CHAPTER THREE

METHODOLOGY

3.1 STUDY AREA

The sampling locations for this research study were Lagos, Ogun, Oyo, Ondo and Osun States in Nigeria. These states fall under the South-Western Geo-political zone of Nigeria. These states were chosen due to their high population status in Nigeria. There is high availability of affordable, ready-to-eat game meat that are usually sold in several designated ‘hotspots’ in the sampling locations. The ‘hotspots’ of game meat are areas that are known specifically for the sales of different types of game meat. Most of these hotspots for the sales of these game meat are not hygienic and have questionable or poor sanitary etiquette, hence, the reason why Lagos, Ogun, Oyo, Ondo and Osun State were chosen as the area of study for this research.

Table 3.1: Table showing the Sample obtained and location

LOCATION	NAME OF ISOLATE	ISOLATE ID		TOTAL
		LAGOS STATE		
Festac	Pangolin	PA_1L_1, PA_2L_1	2	
	Sparrow	SPL_1	1	
	Bird	BIL_1	1	
Oluwo Market	Deer	D_2L_1	1	25
	Bush dog	B_2L_1	1	
	Grasscutter	G_1L_1	1	
	Etu	ETL_1	1	
	Wild Cat	WCL_1	1	
	Atika	ATL_1	1	
	Agbonrin	AGL_1	2	
Epe Fish Market	Antelope	A_1L_1, A_2L_1, A_3L_1,	3	
	Monkey	M_1L_1, M_2L_1	2	
	Rabbit	R_1L_1, R_2L_1	2	
	Bush dog	B_1L_1	1	
	Grasscutter	G_2L_1, G_3L_1, G_4L_1	3	
	Porcupine	PL_1	1	
	Deer	DL_1	1	
		OGUN STATE		
Sango-Ota	Antelope	AN_1S_1, AN_2S_1	2	
	Grasscutter	GRS_1	1	12
	Rabbit	RAS_1	1	
	Bush rat	BUS_1	1	
	Igala	IGS_1	2	
Abeokuta	Antelope	ANA_1	1	
	Rabbit	RAA_1	1	
	Hedgehog	HEA_1	1	
	Guinea fowl	GUA_1	1	
	Alligator	ALA_1	1	
		ONDO STATE		
Oja-Oba Market	Civet Cat	CC_1O_1	1	
	Rabbit	RAO_1	1	9
	Antelope	AN_1O_1, AN_2O_1, AN_3O_1	3	
	Grasscutter	GR_1O_1	1	
Ore		GR_2O_1	1	
	Grasscutter	GUO_1	1	
	Guinea Fowl	CC_2O_1	1	
	Civet Cat			
		OSUN STATE		
Sabo	Hare	HS_1	1	5
	Sese	SS_1	1	
	Antelope	A_4S_1, A_5S_1, A_6S_1	3	
		OYO STATE		
Ibadan	Aparo	API_1	1	
	Eta	ETI_1	1	4
	Esii Tuku	ESI_1	1	
	Guinea Fowl	GFI_1	1	
TOTAL				55

3.2 SAMPLE COLLECTION

Ready-to-eat game meat samples were purchased from the vendors at designated hotspots and collected in sterile zip-lock bags and transported to the laboratory. The game meats were mainly dried cured meat. A total of 55 samples were gotten from different bush meat hotspots across the sampling locations; Lagos, Ogun, Oyo, Ondo and Osun state.

3.3 STERILIZATION

Sterilization of micro-pipette tips, Eppendorf tubes at 121°C in the autoclave and petri dishes, glassware (conical flasks, beakers) at 160°C in the oven was carried out at each phase of work to ensure septic condition and to prevent cross-contamination. The work bench/area was regularly disinfected with 70% alcohol. A flame, provided by the Bunsen burner was always kept on, ensuring the sterility of the air while working.

3.4 MATERIALS AND EQUIPMENTS USED

Materials used: Micro pipette, Micro-pipette tips, Beakers, conical flask, Petri dishes, Eppendorf tubes, Cryovial tubes, Test tubes, test tube racks, filter paper, spatula, inoculating loop, hockey stick.
Equipment used: Autoclave, oven, weighing balance, Stomacher, Incubator (Memmert) Vortex mixer, distiller, water bath.

3.5 PREPARATION OF CULTURE MEDIA

For the isolation, enumeration and identification of *Escherichia coli*, selective and differential media were employed. A Selective media is a media that favours the growth of a specific organism, while inhibiting the growth of other microorganisms that are not the target organism of interest. On the other hand, differential media refers

to a media that allows the growth of more than one microorganism of interest but with morphologically distinguishable colonies.

Mac Conkey agar was used, as the differential agar to differentiate the lactose fermenting bacteria from the non-lactose fermenting bacteria. It produces pink coloured colony of the suspected *Escherichia coli* isolate.

The Sorbitol Mac Conkey agar (SMAC) was used for the presumptive isolation and of the STEC serovar O157:H7. It was prepared by dissolving 50.03g of the powder in 1000ml of water and autoclaved at 121℃ for 15 minutes.

Nutrient agar was used for the identification of total viable count. It was prepared according to the manufacturer's instruction; 28g of Nutrient agar powder in 1000ml of water and autoclaved at 121℃ for 15 minutes.

The Brain Heart Infusion broth (BHI) is a general-purpose media that was used to cultivate and maintain the isolates. It was prepared by dissolving 37g in 1L of water at 121℃.

Buffered Peptone Water (BPW) was used for the primary enrichment of the samples. It contains Peptone at low concentration which provides nutrients for survival of microorganisms. Buffered Peptone water is also very important for the resuscitation of cells that have been injured by processes of food preservation. 0.1% Buffered Peptone Water (BPW) was used for the serial dilution. 0.1% Buffered Peptone water (BPW) was obtained by dissolving 20g of the Peptone Powder in 1000ml of water, then it was autoclaved at 121℃ for 15 minutes.

3.6 SAMPLE PREPARATION

25g of meat was measured aseptically for each bush meat sample using the weighing balance. The 25g of each sample was finely chopped using a sterile knife and then put into a conical flask containing 225ml of 0.1% Buffered Peptone Water. The samples were homogenized in the stomacher for 2 minutes. This is referred to as the primary enrichment.

3.7 SERIAL DILUTION

1000µl (1ml) was pipetted from the primary enrichment of each sample and introduced into the test tube containing 9ml of 0.1 % BPW. To obtain the diluents, 1ml was pipetted from the first dilution factor (10^0) and into the second dilution factor. This process was repeated till the dilution factor of 10^{-6} was attained.

3.8 PLATING

The spread plate technique was used for plating into the prepared Sorbitol Mac Conkey Agar (SMAC) and Mac Conkey Agar (MAC) plates. 100µl (0.1ml) of the 10^{-1} and 10^{-3} diluent factors were inoculated into the Mac Conkey Agar (MAC) and Sorbitol Mac Conkey Agar (SMAC) plates and spread using 100% ethanol. This was done by dipping the hockey stick into the 100% alcohol, flaming with the Bunsen burner and letting to cool off, then spreading the plate to ensure even distribution of the innoculum. Replicates were made for each sample. The plates were then inverted and incubated at 37°C for 18-24 hours.

3.9 SUB-CULTURING

The incubated plates were checked after a duration of about 24 hours for growth. The morphological characteristics such as the shape, colour, colony morphology, elevation, edge and other characteristics were also recorded in detail. A sub-culture is done to purify the isolated bacterial colonies from a mixed bacterial culture. Hence, sub-culturing is done to obtain pure isolates or culture. The Nutrient Agar is used for sub-culturing from the Sorbitol Mac Conkey Agar (SMAC). The Nutrient agar plates are divided into four quadrants and labeled E1 to E4. The process of sub-culturing involves the selection of distinct colonies from the plates for each sample. For *Escherichia coli*, the white and pink colonies are suspected to be *Escherichia coli*; O157 and non- O157 respectively. Two distinct white colonies and pink colonies are selected from each sample plate's diluent factors. The E1 and E2 quadrants is for the white colonies and the E3 and E4 quadrants are for the pink colonies. The sub-

culturing involves streaking a loop full of the selected colonies into each of the designated quadrants using the inoculating loop.

3.10 BIOCHEMICAL TESTS

For the identification of the presumptive isolates, after the morphological characterization further tests were carried out to confirm if the isolates were truly the organism of interest. Below are the tests carried out for the confirmation of the suspected *E. coli* isolates.

3.10.1 GRAM STAINING

Gram staining test is a test done to differentiate bacteria on the basis of their cell wall. This was done to differentiate the bacterial isolates based on the staining property of their cell wall, into either Gram positive or negative, with the use of dyes to enhance the visibility of their cell wall. This was done by making a smear of the selected isolate on a glass slide, it was then heat fixed by passing it over the Bunsen burner flame. The slides were then flooded with Crystal Violet which served as the primary stain for 1 minute and washed off with water. Next, the slides were flooded with Iodine (mordant) and allowed to sit for 1 minute, then washed off with water. The slides were decolorized with 70% alcohol for 20 seconds and rinsed with water. The final step was counterstaining the slides with Safranin for 30 seconds and then rinsed off. On the basis of the Gram Staining, the isolates that retained the colour of the Primary stain (Crystal Violet) after being treated with the 70% alcohol are Gram Positive while the isolates that were decolorized after treatment with alcohol and retained the colour of the Counterstain (Safranin) ranging from pink to red are Gram Negative. The stained slides are dried with blotting paper and immersion oil is applied to the slides and observed under the microscope using the oil immersion objective lens (x100). The observations were documented.

3.10.2 CATALASE TEST

Catalase test is can be used in the identification of Enterobacteriaceae. Therefore, this test was carried out to check the isolate that possess the enzyme catalase, that breaks down Hydrogen Peroxide (H_2O_2) into oxygen and water. On a clean microscopic slide, a loop full of distinct colony from the pure culture plate was picked using the inoculating loop and smeared onto the slide. Few drops of 3% Hydrogen Peroxide was dispensed on the fixed slide and if it produced bubbles which is Oxygen gas, then it means the organism is catalase positive. If there was no production of bubbles then it means the organism is unable to produce the enzyme, hence, Catalase negative.

3.10.3 OXIDASE TEST

This test was carried out to check if the isolates could produce the enzyme oxidase. A filter paper was flooded with the oxidase reagent. A loop full of the isolate was inoculated on the filter paper. A purple coloration after 1 minute indicated that the test was oxidase positive, and colourless (no colour change) indicates oxidase negative.

3.10.4 MOTILITY TEST

The Motility test as the name implies, was carried out to determine if the suspected *E. coli* isolates were capable of moving independently using metabolic energy. A sterile inoculating needle was used to pick the suspected colony and was stabbed into the center of the semi-solid agar in the test tube. A diffuse zone of growth that extends from the line of inoculation indicates that the tested organism is motile.

3.10.5 OXIDATIVE FERMENTATIVE TEST

The Oxidative Fermentative test was carried out to determine if the suspected *E. coli* isolates could metabolize glucose by fermentation or aerobic respiration (oxidatively). 10ml of the oxidative fermentative medium was prepared and autoclaved at 121°C for 15 minutes. 10ml of Glucose was dispensed into Durrham tubes containing 100ml of distilled water. Thereafter, 1ml of the solution containing glucose was dispensed in

the Oxidative fermentative medium, followed by the inoculation of the suspected isolates. A colour change from Green to Orange was observed.

3.10.6 PRESERVATION OF SAMPLES

A loop full of each isolate was inoculated into test tubes containing 5ml Brain Heart Infusion broth (BHI) for 18-24 hours. After this, the solution was homogenized and 750µl was taken from the tube and dispensed into Eppendorf tubes. 750µl of 20% glycerol was added to the Eppendorf tubes, homogenized and stored at -80°C in an ultra-freezer.

3.11 DNA EXTRACTION

DNA extraction is a method used to extract or separate pure DNA from biological samples (Game meat). It separates the pure DNA from cellular membrane, proteins and other cellular components. Hence, DNA extraction was carried out to extract the DNA from microbial cells of the game meat samples, using the Boiling Technique.

3.11.1 MATERIALS USED

The following materials were used in the DNA extraction process. They include: Microbial cells (Preserved Game meat samples), Sterilized distilled water, Micropipette tips, Micropipette, Centrifuge, Heating block, Eppendorf tubes, Autoclave, Brain Heart Infusion Broth (BHI), Vortex mixer, Inoculating loop, Ice.

3.11.2 ACTIVATION OF ISOLATES

- 1ml of pure Brain Heart Infusion broth (BHI) was prepared, and dispensed into 2ml Eppendorf tubes. It was sterilized in the autoclave at 121ºC for 15 minutes.
- After setting it down to cool, 100µl of each of the thawed preserved stock cultures into the various Eppendorf tubes containing 1ml BHI.
- The isolates were incubated at 37ºC for 2 days (48 hours).

3.11.3 PRE-WASHING

- Each of the activated isolates were centrifuged at 5000g for 3 minutes.
- The BHI supernatant was decanted into the waste container, leaving the supernatant at the bottom of the Eppendorf tube.
- 1.5ml of the sterilized distilled water was added into the tubes, they were mixed and centrifuged at 5000g for 3 minutes.
- The supernatant was discarded. 200µl of distilled water was added to the tubes mixed using the Vortex mixer.

3.11.4 BOILING

- The Heating block was turned on and allowed to heat up to boiling temperature (100ºC).
- The Eppendorf tubes containing the pre- washed isolates were carefully sealed and placed in the heating block, then covered with the lid to prevent them from popping out.
- It was allowed to boil for 15 minutes.
- The boiled DNA was placed into ice to cool for 5 minutes.
- It was centrifuged at 7000g for 6 minutes.
- 150µl of the DNA supernatant was carefully transferred into the properly coded fresh Eppendorf tubes.

3.12 POLYMERASE CHAIN REACTION (PCR)

Polymerase Chain reaction (PCR) is a commonly used technique to make billions of copies of DNA from a small sample. It was created by the American Biochemist and Doctor of Chemistry Kary Muler in the year 1985. The extracted DNA samples from each Game meat sample was amplified using the Multiplex Polymerase Chain (PCR) technique. The cocktail mixture was prepared and loaded into the Thermocycler. The Multiplex PCR technique was used to amplify multiple DNA sequences together simultaneously and was carried out in two treatments. The initial denaturation at 95°C for 5 minutes; 35 cycles of 95°C for 2 minutes; 42°C for 30 seconds; and 72°C for 4 minutes, thereafter followed by a final elongation step at 72°C for 10 minutes included in Table 3.2.

3.13 GEL ELECTROPHORESIS

Gel Electrophoresis is a method used to separate and analyze the DNA molecules on the basis of their size and charge. Each DNA was loaded into the perforated agarose gel. The agarose gel was placed in the buffer and current was passed through via the negative terminal. The positive samples migrated towards the positive terminal and was viewed under Ultra Violet light with the aid of gel documentation system.

Table 3.2 Table showing the target genes, virulence factors and primer sequences adapted from Persson (2007).

TARGET GENE	VIRULENCE FACTOR	SEQUENCE (5´-)	FINAL CONCENTRATION(µm)
Human *est*A	STFh	TTTCGCTCAGGATGCTAAACCAG	0.4
		CAGGATTACAACACAATTCACAGCAGTA	
Porcine *est*A	STIp	CTTTCCCCTCTTTTAGTCAGTCAACTG	0.4
		CAGGATTACAACAAAGTTCACAGCAG	
*vtx*1	VT1	GTTTGCAGTTGATGTCAGAGGGA	0.25
		CAACGAATGGCGATTTATCTGC	
*vtx*2	VT2	GCCTGTCGCCAGTTATCTGACA	0.5
		GGAATGCAAATCAGTCGTCACTC	
Eae	Intimin	GGYCAGCGTTTTTTCCTTCCTG	0.15
		TCGTCACCARAGGAATCGGAG	
*elt*A	LTI	AAACCGGCTTTGTCAGATATGATGA	0.45
		TGTGCTCAGATTCTGGGTCTCCT	
*ipa*H	IPaH	TTGACCGCCTTTCCGATACC	0.1
		ATCCGCATCACCGCTCAGAC	
16SrDNA	16SrDNA	GGAGGCAGCAGTGGGGAATA	0.25
		TGACGGGCGGTGTGTACAAG	

Table 3.3: Protocol for Thermocycler

ANALYSIS	STEP	TEMPERATURE(°C)	TIME
1x	Initial denaturation	95	15 min
35x	Denaturation	94	50 sec
	Annealing	57	40 sec
	Polymerization	72	50 sec
1x	Final Polymerization	72	3 min
1x	Hold	4	∞

Table 3.4: Multiplex PCR Components for the PCR Treatment

S/N	REAGENT	INITIAL CONCENTRATION (µm)	FINAL CONCENTRATTION (µm)	V/R(µl)
1	Master mix	5x	1x	2
2	StFp	20	0.4	0.2
3	StRp	20	0.4	0.2
4	*Vtx1*Tf	20	0.25	0.125
5	*Vtx1*tR	20	0.25	0.125
6	*Vtx2*tF	20	0.5	0.25
7	*Vtx2*tR	20	0.5	0.25
8	ipaHF	20	0.1	0.05
9	ipaHR	20	0.1	0.05
10	$MgCl_2$	25	1.5	0.6
11	dH_2O			4.15
12	DNA			

CHAPTER FOUR

RESULTS

4.1 RESULTS

4.1.1 TVC RESULT

The microbial analysis of the isolates for Total viable count (TVC) of Shiga Toxin Producing *E. coli* (STEC) and general *E. coli* gotten from culture on SMAC are shown in Table 4.1. The TVC for the game meat samples were relatively high, starting from 4.3 x 10^6 Cfu/ml which was the Eta to the Monkey with the highest count of 9.5 x 10^6 Cfu/ml, followed by antelope with a TVC ranging from 8.3-8.6 x10^6 Cfu/ml.

4.1.2 BIOCHEMICAL TESTS OF *E. COLI* ISOLATES

Table 4.2 summarizes the biochemical tests carried out on all presumptive *E. coli* isolates. All the samples were negative to Gram staining, Catalase positive, Oxidase negative, Motility positive and fermentative, which are all characteristics of *E. coli*.

Table 4.1: Total Viable Count (TVC) of *E. coli* in the game meat (n=55) from various locations in South western Nigeria.

LOCATION	GAME MEAT	NUMBER OF SAMPLES	TOTAL VIABLE COUNT (Cfu/mL)
Lagos	Pangolin		8.6×10^6
	Quail		4.1×10^6
	Deer		8.1×10^6
	Bush dog		6.4×10^6
	Grasscutter		8.5×10^6
	Etu		5.5×10^6
	Wild cat	25	7.3×10^6
	Atika		6.3×10^6
	Agbonrin		4.5×10^6
	Antelope		8.7×10^6
	Monkey		9.5×10^6
	Rabbit		7.5×10^6
	Porcupine		8.3×10^6
Ogun	Antelope		8.6×10^6
	Grasscutter		8.4×10^6
	Rabbit		7.8×10^6
	Bush rat	12	6.2×10^6
	Igala		6.7×10^6
	Hedgehog		5.2×10^6
	Guinea fowl		4.8×10^6
	Alligator		7.3×10^6
Ondo	Civet cat		7.2×10^6
	Rabbit		7.4×10^6
	Antelope	9	8.8×10^6
	Grasscutter		8.3×10^6
	Guinea fowl		4.4×10^6
Osun	Hare		4.8×10^6
	Sese	5	6.8×10^6
	Antelope		8.6×10^6
Oyo	Aparo		5.5×10^6
	Eta		4.3×10^6
	Esii Tuku	4	7.1×10^6
	Guinea fowl		5.0×10^6
TOTAL		55	

Table 4.2:The results of biochemical tests for presumptive identification of *E. coli* in all isolates.

Sample ID	Gram Staining	Catalase	Oxidase	Motility	Oxidative/Fermentative	Suspected Organism
PA_1L_1	-	+	-	+	F	*E. coli*
PA_2L_1	-	+	-	+	F	*E. coli*
SPL_1	-	+	-	+	F	*E. coli*
BIL_1	-	+	-	+	F	*E. coli*
D_2L_1	-	+	-	+	F	*E. coli*
B_2L_1	-	+	-	+	F	*E. coli*
G_1L_1	-	+	-	+	F	*E. coli*
ETL_1	-	+	-	+	F	*E. coli*
G_2L_1	-	+	-	+	F	*E. coli*
BUS_1	-	+	-	+	F	*E. coli*
RAA_1	-	+	-	+	F	*E. coli*
HEA_1	-	+	-	+	F	*E. coli*
GUO_1	-	+	-	+	F	*E. coli*
SS_1	-	+	-	+	F	*E. coli*
ETI_1	-	+	-	+	F	*E. coli*

Note: '+' indicates positive result, '-' indicates negative result and 'F' indicates Fermentative.

4.1.3 PREVALENCE OF SHIGA TOXIN *E. COLI* IN GAME MEAT

The prevalence of the game meat samples (n=55) show that Antelope and Grasscutter are the most prevalent (100%).

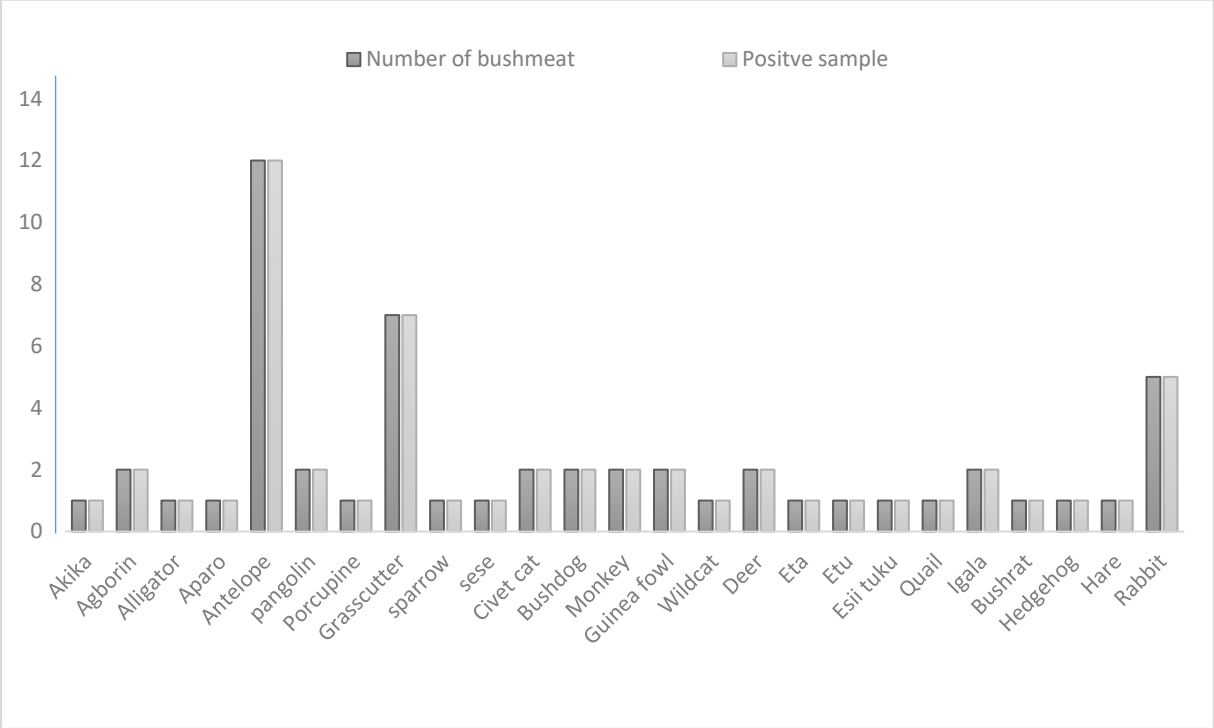
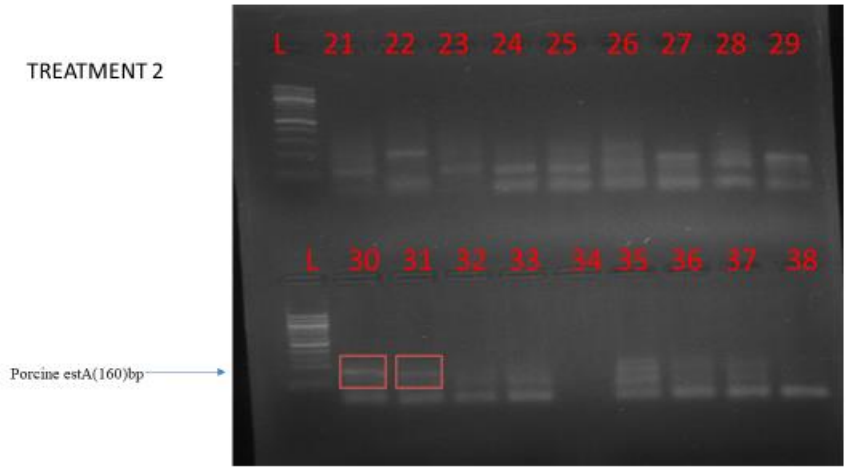


Figure 4.1: The prevalence of presumptive Shiga toxin producing *E. coli* in game meat (n=55).



TREATMENT 1: Human *estA* (StFh and StRh primers) – 151bp
vtx1 – 260bp
vtx2 – 420bp
ipaH – 647bp

TREATMENT 2: Porcine *estA* (StFp and StRp primers) – 160bp
eae – 377bp
eltA – 479bp

Plate 4.1: Agarose gel electrophoresis image of Multiplex-PCR products (*Human estA, Porcine estA, vtx1, vtx2,ipaH, eae, elta*). Lane L: marker (100-bp ladder), lane 30 and 31: *E. coli* isolate (Porcine estA).

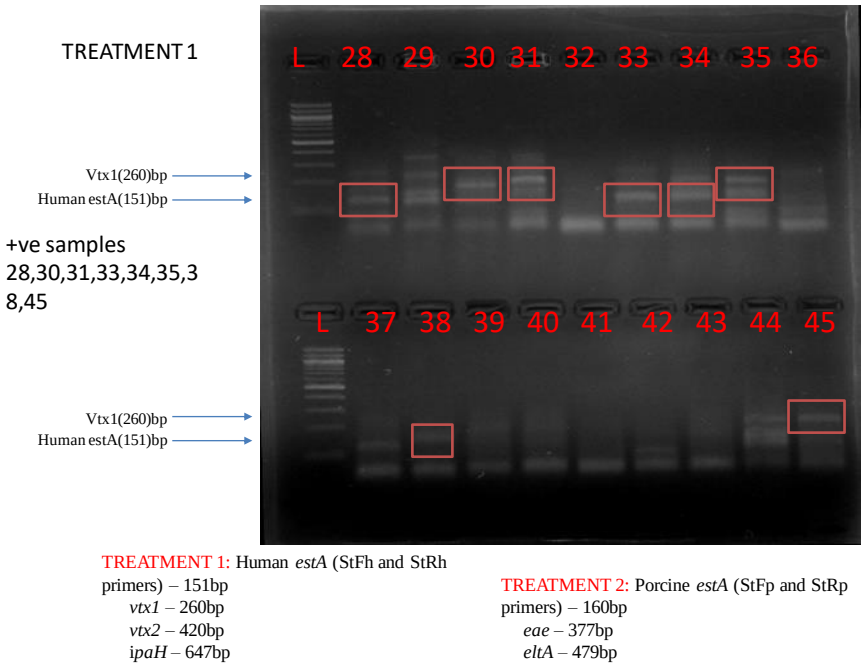


Plate 4.2: Agarose gel electrophoresis image of Multiplex-PCR products (*Human estA, Porcine estA, vtx1, vtx2, ipaH, eae, elta*). Lane L: marker (100bp-ladder). Lane 28, 33, 34, 38 (Human estA), Lane 30, 32, 35, 45 (*Vtx1*).

4.1.4 PREVALENCE OF POSITIVE SAMPLES ACCORDING TO LOCATION

All the game meat samples were positive for *E. coli*, however, the Table 4.3 shows the number of Positive Samples of the *E. coli* gene of interests (*Vtx1*, Human and Porcine estA). Ogun state has the highest number of positive samples (12.7%), Ondo and Osun States with the lowest number of positive samples (1.8%).

Table 4.3: Number of Positive samples and their location

LOCATION	LAGOS	OGUN	OYO	ONDO	OSUN
NO. OF POSITIVE SAMPLES	3	7	2	1	1

CHAPTER FIVE

DISCUSSION

5.1 DISCUSSION

Escherichia coli is ranked as the most studied bacterial pathogen due to the impact on public health (Pokharel *et al*., 2020). The existence of several game meat species and availability at relatively affordable rates further encourages the sales, hence, consumption of game species in Nigeria. Shiga Toxin Producing *E. coli* (STEC) has majorly been implicated in cattle and Sheep (Colleloet *al*., 2015).

This research aimed at isolating and detecting *E. coli* from ready-to-eat game meat samples sold at various game meat hotspots in the South West region of Nigeria.

The game meat samples were investigated to assess the safety in consuming game meat due to the insufficient knowledge on the microbiological quality of game species in Nigeria.

In all the game meat samples, *E. coli* was suspected, *E. coli* is used as an indicator of fecal matter, meaning that the contamination of the game species is probably due to fecal contamination, poor sanitary conditions, improper handling or even storage lapses.

The Total Variable Count (TVC) was also used an indicator to assess the microbial safety of the game meat samples. The TVC of the game meat samples ranged from 4.3 to 9.5 x 10^6 $\log_{10}$Cfu/ml shows that they all possessed relatively high microbial load but still tolerable as it does not exceed the Food and Organization (FAO) limit of 10^5 Cfu/ml (FAO, 2005). Despite the fact that the TVC does not assess the microbial quality of the food, it is used as an indicator of processing, handling, storage of the sample.

The PCR results of the presumptive *E. coli* (n=55) show that all the samples for *E. coli* are positive, however, the target *E. coli* genes of interest identified was the Human estA, Porcine estA, and *Vtx1*; 12.7%, 3.6% and 9.1% respectively. Antelope was the most implicated game meat specie. A recent study on beef products in Lagos state, also confirmed the presence of STEC in ready-to-eat beef products using Multiplex PCR (Fayemi*et al*., 2021).

Shiga Toxin Producing *Escherichia coli* (STEC) has been known for causing unpredictable and deadly outbreaks of infectious diarrhea, Hemorrhagic Uremic Syndrome and hemorrhagic colitis (Vallance *et al*., 2002). Therefore, the presence of STEC in game meat that is now being commonly consumed is a health risk which could potentially be a hazard.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS AND RECOMMENDATIONS

Based on this research finding, the presence of pathogenic *E. coli* and Shiga toxin producing *E. coli* (STEC) was suspected and detected in few of the game meat species. The frequency of the contaminated samples albeit low, still indicates that there are sanitary lapses in the processing of these ready-to-eat game meat. These game meat species are casually sold, and the presence of STEC in them is an indication that the microbiological safety is compromised, hence, a risk to the public health. There should be strict enforcement of Hazard and/or risk-based food safety protocols to prevent or mitigate the occurrence and incidence of pathogenic microorganisms in game meat by appropriate food safety and Public health authorities.

Periodical quality audits to monitor the Processes used in handling and processing game meat and its products. Generally, the game meat must be properly and thoroughly cooked to eliminate microorganisms. Surveillance systems should be put in place to track and the monitor occurrence of foodborne illnesses and/or diseases as a result of consuming game meat.

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