PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN CATTLE IN KADUNA AND SOKOTO STATES, NIGERIA

BY

LUGA, IGBAKURA INNOCENT
(DVM (ABU) 1986; M.Sc (ABU) 1995)
Ph.D /VET-MED/47549/2004-05

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AUGUST, 2007

DECLARATION

I declare that the work in the dissertation entitled "Prevalence and Antimicrobial Susceptibility of Shiga toxin-producing *Escherichia coli* in Cattle in Kaduna and Sokoto States, Nigeria" has been performed by me in the Department of Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria and the National Veterinary Research Institute, Vom,Nigeria, under the supervision of Professors Jacob K.P. Kwaga, Veronica J. Umoh and Ikwe Ajogi.

This dissertation is the result of years of research and includes nothing which is the outcome of work done in collaboration with other researchers. The information derived from literature has been duly acknowledged in the text and list of references provided. The assistance obtained from other individuals during the course of the study has been acknowledged and directly referred to in the text. No part of this dissertation was previously presented for another degree or Diploma at any University.

Luga, Igbakura Innocent August 29, 2007

_____	_____	_____
Name of Student	**Signature**	**Date**

CERTIFICATION

This dissertation entitled, **"PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN CATTLE IN KADUNA AND SOKOTO STATES, NIGERIA"** by Luga, Igbakura Innocent meets the regulations governing the award of the Degree of Doctor of Philosophy of Ahmadu Bello University, Zaria, Nigeria and is approved for its contribution to scientific knowledge and literary presentation.

_____ _____

Professor Jacob K.P. Kwaga, Date
Chairman, Supervisory Committee,
Department of Veterinary Public Health and
Preventive Medicine,
Ahmadu Bello University, Zaria, Nigeria.

_____ _____

Professor Veronica J. Umoh, Date
Member, Supervisory Committee,
Department of Microbiology,
Ahmadu Bello University, Zaria, Nigeria.

_____ _____

Professor Ikwe Ajogi Date
Member, Supervisory Committee,
Department of Veterinary Public Health and
Preventive Medicine,
Ahmadu Bello University, Zaria, Nigeria.

_____ _____

Dr. Daniel H.M. Du-Sai Date
Head, Department of Veterinary Public Health
and Preventive Medicine,
Ahmadu Bello University, Zaria, Nigeria.

_____ _____

Dean, Post Graduate School,
Ahmadu Bello University,
Zaria, Nigeria

D**EDICATION**

To my wife, Aseer

my son, Ak and my daughter, Ud.

You are my treasure.

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ABSTRACT

A total of 3000 samples comprising of 1800 faecal,400 unpasteurized milk,300 abattoir effluents, 300 water,20 minced meat and 20 cheese were collected and enriched in modified tryptone soya broth (MTSB) supplemented with novobiocin (Oxoid, Basingstoke England) and plated on cefixime sorbitol – MacConkey (CR-SMAC) agar (Oxoid, Basingstoke England). Typical colourless colonies were biochemically tested using standard methods. Isolates biochemically identified as *E. coli*, were presumptively identified using Remel Wellcolex *E. coli* O157:H7 kit (Remel Europe, UK) and a reference strain of *E. coli* O157:H7 (EDL 933). Shiga toxin production was assayed using the Verotoxin *E. coli*–Reverse Passive Latex Agglutination (VTEC-RPLA) kit (Oxoid, Bassingstoke, England). All STEC O157 strains were tested for haemolytic activity by culturing in brain heart infusion (BHI) agar containing 7% human blood. Molecular assays were carried out using four sets of primers targeting $rfbE_{O157}$, $fliC_{H7}$, stx_{2d} *and* stx_1 genes in all the confirmed isolates of *E. coli* O157:H7 from faecal and bulk milk samples and all *E. coli* O157:H- from bulk milk and 13 from cattle faeces. Antimicrobial resistance testing was carried out on EHEC STEC O157 by the standardized single disk diffusion method on Mueller – Hinton agar with EHEC EDL 933, as the control organism and a panel of 16 antimicrobial agents (Oxoid Basingstoke, England). The occurrence of enterohaemorrhagic STEC O157:H7 in healthy dairy, nomadic white Fulani cattle and unpasteurized bulk milk was confirmed by multiplex-PCR, duplex-PCR and PCR. The prevalence of faecal excretion of *E. coli* O157:H7 by multiplex-PCR was 0.6% (10/1800). This prevalence was significantly associated with season (Fisher's exact test, $p=0.030$), age (Fisher's exact test, $P<0.040$) and management type (Fisher's exact test,

p=0.034). However the prevalence by multiplex-PCR was not significant for diarrhea (Fisher's exact test, p=0.532), breed (Fisher's exact test, p=0.440), water quality (Fisher's exact test, p=0.627).Twenty one (75%) out of the 28 *E. coli O157* strains tested showed resistance to at least one of the antimicrobial agents. Resistance to sulphonamides (64%) was highest in all classes of antimicrobial agents tested, followed by resistance to tetracycline (36%), aminoglycosides (21%), β-lactams (21%) and to quinolones (18%). Among the multiplex – PCR confirmed isolates of faecal origin, resistance by class of antimicrobial agent was highest in sulphonamides (80%), followed by tetracycline and β-lactams (30%), aminoglycosides and quinolones (20%). Twenty-one resistance patterns were detected, including six multi-drug resistant (MDR) phenotypes. This data suggests that selection pressure imposed by the use/misuse of antimicrobial agents in veterinary practice and/or as prophylaxis in the animal production environment in Northwestern Nigeria may have generated a pool of MDR enterohaemorrhagic STEC O157:H7 in the cattle population in this zone. However, the resistance to nalidixic acid suggests a possible role for novel resistance mechanisms in *E. coli* from cattle in Northwestern Nigeria. To our knowledge, this is the first report of the detection in *E. coli* of the $rfbE_{O157}$, $fliC_{H7}$, stx_{2d} *and* stx_1 genes from bovine isolates in Northwestern Nigeria and the stx_{2d} gene from cattle isolates of *E. coli* O157:H7.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Statement of the Problems

Shiga (Shiga-like) toxin-producing *Escherichia coli* O157:H7 (STEC O157:H7), also referred to as verotoxin (verocytotoxin) – producing *Escherichia coli* O157:H7 (VTEC O157:H7) is an important food borne pathogen that has emerged as a major cause of diarrhea in humans (Riley *et al.*, 1983; Karmali, 1989; Griffin and Tauxe, 1991; Barret *et al.*, 1994; Armstrong *et al.*, 1996; Allison *et al.*, 2000). Dairy cattle have been implicated as the primary reservoir of *Escherichia coli* O157:H7 (Chapman *et al.*, 1997; Hancock *et al.*, 1998; Heuvelink *et al.*, 1998a, b; Paiba *et al.*, 2002, 2003) and most outbreaks of *E. coli* O157:H7 infections usually have a bovine origin (Armstrong *et al.*, 1996).

Escherichia coli O157:H7 is transmitted to humans by ingestion of contaminated food and water, directly from person-to-person and occasionally through occupational exposure (Armstrong *et al.*, 1996). The symptoms of the infection in man are: mild uncomplicated diarrhea and haemorrhagic colitis (HC). Complications associated with *E. coli* O157:H7infection in man include haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (http://www.about-ecoli.com/main.html; Wang *et al.*, 2002; Bidet *et al.*, 2005).

Infections caused by *Escherichia coli* O157:H7 have been widely reported since it was first described as enterhaemorrhagic and associated with diarrhea and haemorrhagic colitics in humans by Riley *et al.* (1983). Epidemics associated with the pathogen have

been described worldwide (Fukushima *et al.*, 1999; Allison *et al.*, 2000; Liesegang *et al.*, 2000; Effler, *et al.*, 2001). Epidemic and sporadic strains have been characterized in North America (Preston *et al.*, 2000; Davis *et al.*, 2003; Noller *et al.*, 2003), the United Kingdom (Willshaw *et al.*, 1997; Allison *et al.*, 2000), Germany (Liesegang *et al.*, 2000), Japan (Izumiya, *et al.*, 1997; Watanabe *et al.*, 1999), and Swaziland and South Africa (Effler *et al.*, 2001).

Isolation of the organism has been reported from many countries in Africa including South Africa (Browning *et al.*, 1990), Uganda (Kaddu-Mulindwa *et al.*, 2001), Egypt (Abdoul-Raouf *et al.*, 1996), Cameroon (Germani *et al.*, 1998), Cote d'Ivoire (Dadie *et al.*, 2000) and in Nigeria (Akinyemi *et al.*, 1998; Okeke *et al.*, 2000; Olorunshola *et al.*, 2000; Smith *et al.*, 2003).

1.2 Justification

The control of *E. coli* O157:H7 is mainly centered on animals and the farm environment. The two-fold aim is to ensure that the pathogen does not enter the food supply and to assess factors contributing to the emergence and survival of the organism in animals and the environment (Effler *et al.*, 2001).

In Nigeria there is dearth of literature on the status of animals, the farm and the farm environment as epidemiological factors in the handling of *E. coli* O157:H7 infections in animals. Pioneering work on Shiga toxin-producing *E. coli* (STEC) in Zaria Northwestern Nigeria by Tekdek (1994) reported the occurrence of O? O8, O15, O75,

O26 and O6 serogroups in calves. Efforts during outbreaks in humans have been concentrated on hospital-based cases. For example, Akinyemi *et al*. (1998) studied patients with acute gastroenteritis in Lagos. Okeke *et al.* (2000) characterized strains from children with diarrhea in southwestern Nigeria. Olorunshola *et al.* (2000) reported the prevalence of *E. coli* O157:H7 in patients with diarrhea on admission in Lagos Mainland Hospital, Yaba and Lagos University Teaching Hospital (LUTH). Raji *et al.* (2003) have published a review of *E. coli* O157:H7 and the African situation. There is a lack of information on the role played by animals, the farm and the farm environment in the epidemiology of infections caused by the pathogen in Nigeria.

Infection with *E. coli* O157:H7 can lead to death in man due to haemorrhagic colitis (HC) and sequelae such as HUS and TTP. Studies have suggested that initiation of antibiotic therapy early in the stages of the infection was able to prevent the disease progression to HUS (Fukushima *et al.*, 1999; Ikeda *et al.*, 1999; Shiomi *et al.*, 1999). HUS is a severe condition with no specific treatment. Most patients showing the syndrome require prolonged clinical and outpatient treatment (Slutsker *et al.*, 1997). The emergence and dissemination of antimicrobial resistance in STEC O157:H7 may complicate future therapeutic options that are being developed such as the administration of a chemically synthesized analog of Stx receptor Gb3 in an effort to absorb Stx and curtail the toxin induced sequelae (Armstrong *et al.*, 1995; Nataro and Karper, 1998).

This has prompted extensive studies on antibiotic resistance of *E. coli* O157:H7 even though antimicrobial treatment for STEC infections is still regarded as controversial

(Igarashi *et al.*, 1999; Proulx *et al.*, 1992; Yoh and Honda, 1997). The antimicrobial resistance of the pathogen has been studied to elucidate its molecular basis (Zhao *et al.*, 2001), to understand interactions and strategies used by the pathogen in infection (Martinez and Baquero, 2002) and to obtain antimicrobial resistance patterns in designated localities. The antibiotics tested are usually those commonly used in veterinary medicine and antibiotics employed mainly in humans (Babak *et al.*, 2005; Mora *et al.*, 2005; Shroeder *et al.*, 2002).

1.3 Objectives of the Study

The objectives of this study therefore were to:

1. Isolate and presumptively identify *E. coli* O157:H7 from Cattle, Abattoir effluents, Water intended for Cattle consumption, Imported Mince meat and Cheese in Kaduna and Sokoto States.
2. Detect any Shiga toxins produced and Characterize the Isolates by Molecular methods to detect $rfbE_{O157,}$ $fliC_{H7,}$ *stx2* and *stx1* Genes.
3. Determine the Prevalence and Distribution of *E. coli* O157:H7 from Cattle based on Serological and Molecular methods.
4. Determine the Antimicrobial Susceptibility of the Isolates by the Standardized Single Disk Diffusion method on Mueller-Hinton agar.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical Background

E. coli O157:H7 was identified for the first time at the Centers for Disease Control (CDC), United States of America (USA) in 1975. However, it was not until seven years later, in 1982 that *E. coli* O157:H7 was conclusively determined to be an aetiologic agent of an enteric disease in Man. Specifically, in 1982, following outbreaks of food-borne illness in the USA that involved several cases of bloody diarrhea, *E. coli* O157:H7 was firmly associated with haemorrhagic colitis (Riley *et al.*, 1983).

Shiga toxin-producing *E. coli* O157:H7 (STEC O157:H7) is the most important recently emerged food-borne pathogen (Paton and Paton, 1998; FSAI, 1999; Blanco *et al.*, 2004). Most outbreaks of STEC infections have been attributed to *E. coli* O157:H7 (Fukushima *et al.*, 1999; Griffin and Tauxe, 1991). Since its association with clinical disease in humans in 1982, the zoonotic pathogen has generated a lot of interest. It is listed by many authorities as an emerging infectious agent (Armstrong *et al.*, 1996), and the Emerging Infectious Diseases Journal (http//www.cdc.gov/eid/ncidod/E.D/trans.htm). Perna *et al.* (2001) reported the genome sequence of enterohaemorrhagic *E. coli* O157:H7. The organism has been one of the reasons for symposia and seminars: International Conference on Emerging Infectious Diseases 2006 (http://www.iceid.org) and International Symposium on Emerging Zoonoses, Medical and Veterinary Partnerships to Address Global Challenges, (http://www.isezconference.org). It has been the subject of reviews, books and diverse publications, including Survival of *Escherichia coli* O157:H7

in feces from corn and barley-fed steers (Bach *et al.*, 2005); Prevalence of verotoxin producing *Escherichia coli* (VTEC) and *E. coli* O157:H7 in French pork (Bouvet *et al.*, 2002); Infection by verocytotoxin-producing *Escherichia coli,* (Karmali, 1989). The occurrence of *E. coli* O157:H7 in South African water sources intended for direct human consumption (Muller *et al*., 2001). Survival and Growth of *Escherichia coli* O157:H7 in unpasteurized and pasteurized milk, (Wang *et al.*, (1997). A therapeutic agent with oriented carbohydrates for treatment of infections by shiga toxin-producing *E. coli,* (Nishikawa *et al.*, 2002) and Identification and characterization of integrons-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates, (Zhao *et al.*, 2001). *Escherichia coli* O157:H7 is now recognized as the most common STEC (Levine, 1987; Effler *et al.,* 2001; Kaper *et al*., 2004).

2.2 Classification of *E. coli* O157:H7

Escherichia is the type genus of the family *Enterobacteriaceae* and *E. coli* is the type species of the genus. It belongs to the coliform group of organisims. The other coliforms are *Enterobacter, Citrobacter* and *Klebsiella* species (Coghlan *et al.,* 1975)

There are so many different strains of *E. coli,* so microbiologists have classified the bacterium into more than 170 serogroups. Within each serogroup, there are one or more serotypes. The serotypes are based on somatic (O) antigen and flagellar (H) antigen. For instance, 0126:H27 is a serotype of *E. coli* (the somatic antigen is 0126 and the flagellar antigen is H27), 0126:H – is another serotype, 0126 signifying the particular serogroup to

which these two serotypes belong, and O157:H7 and O157:H- belong to the O157 serogroup (http://www.about-ecoli.com/main.html

Escherichia coli O157:H7 is an enterohemorrhagic *E. coli* (EHEC) (Riley, 1983; Levine, 1987); and EHEC is one of six pathotypes of *E. coli* that cause enteric diarrheal infections (Marrs *et al.*, 2005), the others are, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC) and a seventh pathotype has been described for urinary tract infections (UTI), called uropathogenic *E. coli* (UPEC) (Marrs *et al.*, 2005). Each pathotype causes disease using different combinations of virulence factors, with different molecular pathways and in some but not all cases resulting in disease symptoms that can be distinguished from each other. They also vary in their somatic and flagellar (O:H) antigens. The designations are useful for therapeutic and vaccine development purposes (Kaper *et al.*, 2004).

Several strains of *E. coli* are known as verotoxin (verocytotoxin) – producing *E. coli* (VTEC). This group produces a toxin that was named vero-toxin because it was cytopathic for a line of African green monkey kidney cells (Vero cells). This toxin was subsequently termed Shiga-like (Shiga) toxin when it was shown to be similar to Shiga toxin previously associated with *Shigella dysenteriae* 1 (O'Brien and LaVeck, 1983).

2.3 Virulence Determinants (Factors) and Clinical Signs of *E. coli* O157:H7 in Man

Dose consideration for the establishment of an infection with *E. coli* O157:H7 is not important because two cells are enough to cause disease (Coia, 1998; Willshaw *et al.*, 1994). Upon ingestion of the organism, it localizes in the intestines and causes inflammation of the intestinal wall. This inflammatory reaction is caused by the virulence determinants of the organism and is believed to be responsible for the clinical signs of the infection. The virulence factors produced are; Shiga toxin 1 (Stx1), Stx2 or combinations of these toxins, enterohemorrhagic *E. coli* hemolysin and intimin involved in the attaching and adherence phenotype. These factors are encoded for by stx_1, stx_2 EHEC *hlyA* and *eaeA* genes respectively (Cagney *et al.*, 2004). The O157 antigen is encoded for by *rfbE* gene and the *fliC* gene encodes the H7 antigen (Wang *et al.*, 2002; Blanco *et al.*, 2003). Shiga toxins (Stx) are recognized as the main mechanism by which *E. coli* O157:H7 expresses its virulence in man (Karmali,1989), and studies on the organism often target the detection of Stx1 and 2 genes as the virulence determinants (Zhao *et al.*, 1995; Cagney *et al.*, 2004).

Although *Stx1* is relatively homogeneous, five subtypes of *Stx2* have been identified, and these include *Stx2*, *Stx2c* (Ito *et al.*, 1990; Paton *et al.*, 1992; Paton *et al.*, 1993; Lindgren *et al.*, 1994); *Stx2d* (Pierard *et al.*, 1998); *Stx2e* (Gyles *et al.*, 1998; Marques *et al*., 1987) and Stx2f (Gannon *et al.*, 1990; Schmidt *et al.*, 2000). Indeed based on both restriction fragment length polymorphisms (RFLP) of the B-subunit encoding the DNA fragments obtained by PCR and digoxigenin oligonucleotide labeling, DNA probes specific for Stx_2 B-subunit genes encoding *Stx2* and *Stx2c* and *Stx2d* have been further confirmed (Ito *et*

al., 1990; Tyler *et al.*, 1991; Thomas *et al.*, 1993; Gannon *et al.*, 1997; Meng *et al.*, 1997; Meng *et al.*, 1998; Pierard *et al*, 1998; Hu *et al.*, 1999; Feng and Monday, 2000; Fratamico *et al.*, 2000; Pass *et al.*, 2000). Furthermore, 11 variants with distinct genes have been identified from the subtypes (Bertin *et al.*, 2001; Gobieus, *et al.*, 2003). The stx_2 genes have been reported to have high homology in their sequences and are often haboured by the same organism (Zhang *et al.*, 2005).

In addition the presence of the ehl_1 gene, which encodes an enterohemolysin that is unrelated to the product of the EHEC *hlyA* gene, has been associated with a severe outbreak of *E. coli* disease among neonates (Chinen *et al.*, 2002).

The syndrome induced by infection with *E. coli* O157:H7 in humans include watery diarrhoea and haemorrhagic colitis (HC) (Levine, 1987). Typically there is a sudden onset of abdominal pain and severe abdominal cramps, followed within 24 hours by diarrheoa which may become grossly bloody, termed haemorrhagic colitis (HC). Vomiting may occur but there is usually no fever. The incubation period for the disease is 3 to 9 days. The duration of illness is about a week and the disease resolves without any long-term problems. Most individuals recover completely within two weeks with good medical attention (http://www.about-ecoli.com/main.html).

Although most infected people recover from an *E. coli* O157:H7 infection, about 5 – 10% of infected individuals develop haemolytic uraemic syndrome (HUS) as a sequelae to the infection. The presence of serum antibodies to Stx suggests that it enters the circulation.

Binding of Stx to vascular endothelial cells within the CNS or kidney initiates neurologic complications such as HUS. The risk of HUS is greater with Stx2 expression by *E. coli* O157:H7 than Stx1. HUS is a severe life-threatening complication. It was first described in 1995 and is now recognized as the most common cause of kidney failure in childhood. (http://www.about.ecoli.com/main.html). *Escherichia coli* O157:H7 is responsible for over 90% of reported cases of HUS. In fact some researchers believe that *E. coli* O157:H7 is the sole causative agent of HUS in children. Among survivors of HUS, up to 5% may develop kidney disease. The other 5% may experience neurological or pancreatic problems which significantly impair quality of life. An accurate lifetime prognosis is not available since the longest available follow-up studies of HUS are about 23 years. HUS is less common in adolescents and much more common in younger children. (hhp://www.about-ecoli.com/main.html).

Thrombotic thrombocytopenic purpura (TTP) is a clinical syndrome defined by the presence of thrombocytopenia and microangiopathic haemolytic anaemia. Generally called "adult HUS", *E. coli* O157:H7 has been associated with the mechanism inducing endothelial cell damage in TTP. The damage triggers a cascade of biochemical events that ultimately leads to the characteristic feature of TTP, widespread dissemination of hyaline thrombi, which occlude terminal arterioles and capillaries of organs especially the heart, brain, kidneys, pancreas and adrenals glands. Multi-system abnormalities result with the clinical manifestation of a syndrome (http://www.about-ecoli.com/main.html; Ostroff, *et al.*, 1989).

2.4 Treatment of *E. coli* O157:H7 Infection and sequelae in Man

There is no specific therapy for *E. coli* O157:H7 infection. Good supportive care is recommended by most authorities such as close attention to rehydration and nutrition because in most individuals, the intestinal illness lasts about one week and resolves without any long-term complications (http:/www.about-ecoli.com/main.html).

The use of antimicrobials in the management of *E. coli* O157:H7 infection is currently one of the most contentious areas in paediatrics today. This is because of the possible role of the agents in the natural history of the infection (Shroeder *et al.*, 2002; Mora *et al.*, 2005). Antimicrobials may lyse the cell wall of the bacteria thereby liberating Shiga toxins (Walterspiel *et al.*, 1992 Wong *et al.*, 2000). Zhang *et al.*, (2000) reported that quinolone antibiotics induce Shiga toxin-encoding bacteriphages and Shiga toxin production. These authorities recommend the non-use of antimicrobials for treating STEC O157:H7 infection. The counter thought is that infection with STEC O157:H7 could be life threatening mainly due to complications such as HUS and TTP. This has been widely reported during epidemics mainly in Japan, the USA and Southern Africa (Fukushima *et al.*, 1999; Allison *et al.*, 2000; Effler *et al.*, 2001). Studies and experience during these massive epidemics have suggested that initiation of antibiotic therapy early in the stages of the infection was able to prevent the disease progression to HUS (Fukushima *et al.*, 1999; Ikeda *et al.*, 1999; Shiomi *et al.*, 1999). Effler *et al.* (2001) pointed out that patients developed HUS without a history of antibiotic use. Moreover, patients showing the syndrome require prolonged clinical and outpatient treatment (Slutsker *et al.*, 1997). The controversy over antimicrobial treatment for STEC O157:H7

infection is still unresolved especially in children under two years of age (Yoh and Honda, 1977; Proulx *et al.*, 1992; Igarashi *et al.*, 1999). Antibiotic therapy is left to attending physicians. Kanamycin and fosfomycin have been extensively used in Japan (Fukushima *et al.*, 1999). Quinolones especially ciprofloxacin and norfloxacin have also been used (MOHWJ, 1996; Zhang *et al.*, 2000).

2.5 Identification of *E. coli* O157:H7

Identification of *E. coli* O157:H7 by molecular methods is an ever expanding multidisciplinary area. For instance, genomic DNA patterns generated by pulsed-field gel electrophoresis (PFGE) are highly specific for most strains of bacteria and have significant value in epidemiologic investigations of infectious disease outbreaks. Unfortunately, cost, specimen processing and problems associated with a developing economy limit the practical application of this and other technologies as real time epidemic tools (Gautom, 1997).

2.5.1 *Cultural Characteristics*

The majority of *Escherichia coli* O157:H7 do not or ferment D-Sorbitol slowly. Sorbitol-MacConkey (SMAC) agar was developed to take advantage of this phenotypic characteristic by substituting the carbohydrate sorbitol for lactose in MacConkey agar and the medium is indicated for isolation of *E. coli* O157:H7, since *E. coli* O157:H7 rapidly ferments lactose and is indistinguishable from most other *E. coli* on traditional lactose-containing media such as MacConkey agar. *Escherichia coli* O157:H7 is sorbitol-negative and grows as colorless colonies on SMAC agar (March and Ratnam, 1986).

2.5.2 *SMAC Agar Supplements*

These include cefixime, tellurite and rhamnose and rely on phenotypic characteristics of *E. coli* O157:H7 such as inability to ferment the carbohydrates and tolerance to tellurite. The commonly used ones are cefixime-sobitol MacConkey (CR-SMAC) agar, cefixime-tellurite SMAC agar (CT-SMAC) and CT-SMAC agar supplemented with rhamnose (CTR-SMAC) agar. They have the advantage of being designed specifically to detect *E. coli* O157:H7. Tellurite is used with immunomagnetic separation protocol for culturing as CT-SMAC agar (Karch *et al.*, 1996; Cagney *et al.*, 2004). CR-SMAC is used for direct plating and enumeration with or without an enrichment phase (Cagney *et al.*, 2004).

2.5.3 *Transport Media for E. coli O157:H7*

Transport media are indicated so that the organism to be isolated is not injured and is recoverable from the samples. Zhao *et al.*, (1995) used Cary-Blair medium to ensure viability of the organisms from feacal samples of dairy cattle. Another transport medium is Stuart's Transport medium. Transport media are not routinely used for the transportation of samples for isolation of *E. coli* O157:H7. The usual transportation method has been described by Cagney *et al.* (2004): transportation in cooler boxes at 4 ± 1^0C and examination within 64 hours, usually within 24 hours (Bouvet *et al.*, 2002).

2.5.4 *Enrichment Media for E. coli O157:H7*

The common format for isolation of *E. coli* O157:H7 from faeces, food and environmental samples is to use a selective/differential agar usually SMAC. Most individuals and laboratories include an enrichment phase. The most common enrichment

media used are tryptone soya broth preparations marketed under various manufacturers. (Zhao *et al.,* 1995; Islam *et al.,* 2005). Al-Gallas *et al.* (2002) used an enrichment medium containing 1% tryptone in Tunisia to isolate *E. coli* from meat and dairy products. Bach *et al.* (2005) enriched cattle faeces in tryptic soy broth to estimate the survival of *E. coli* O157:H7 in faeces. Buffered peptone water has also been used (Bouvet *et al.,* 2002, Paiba *et al.,* 2003). The enrichment media are often supplemented with antibiotics to improve the isolation rate. The supplements used include; nalidixic acid (Bach *et al.*, 2005), ampicillin (Islam *et al.*, 2005) and novobiocin (Zhao *et al.*, 1995)

2.5.5 *4-Methylumbellferyl-β-D glucoronide (MUG) Supplement*

Escherichia coli O157:H7 strains can be tested for the enzyme β–glucoronidase using agar or broth medium, containing the substrate 4-methylumbellferyl-β-D- glucoronide (MUG). When MUG is cleaved by this enzyme, a fluorescent product is produced that is detectable with long-wave ultraviolet (UV) light. Unlike approximately 92% of *E. coli* , *E. coli* O157:H7 and *E. coli* O157:NM strains that produce Stx lack the enzyme and are MUG negative. For this reason some laboratories use MUG assay in conjunction with sorbitol fermentation on SMAC agar for screening for VTEC O157 (http://www.biosynt.com)

2.5.6 *5-Bromo-4-chloro-3-indoxyl-beta-D-glucoronic acid, sodium salt, (X-β-D-glc UA. Na or BCIG) supplement*

The enzyme β-glucuronidase also reacts with the enzyme substrate 5-bromo-4-chloro-3-indoxyl-beta-D-glucoronic acid sodium salt (X-β-D-glc UA.Na or BCIG) to produce a

deep blue colour seen in colonies of most *E. coli.* Verotoxin-producing *E. coli* O157:H7 lacks the enzyme and the colonies remain straw or colourless (http://www.biosynth.com).

2.5.7 *Biochemical Characteristics*

Escherichia coli is urease negative, citrate negative, indole positive, H_2S negative from triple sugar iron (TSI) agar, H_2S negative in sulphide indole motility (SIM) medium; produces an acid/acid reaction with gas production using TSI agar. The organism is mostly motile using SIM medium, methyl red positive, Voges-Proskauer negative, phenyalanine negative, produces gas from glucose fermentation, and acid reaction from fermentation of lactose, mannitol, maltose, arabinose, trehalose and an acid negative reaction from fermentation of sorbitol and rhamnose (Coghlan *et al.,* 1975)

Indole, methyl red, Voges-Proskauer and citrate (IMViC) reactions are biochemical tests used to preliminarily differentiate *E. coli* from *Klebsiella Enterobacter* and *Citrobacter* which together with *E. coli* are termed coliforms (Coghlan, 1975).

2.5.8 *Presumptive Identification of E. coli O157:H7 by Remel Wellcolex Test*

Sorbitol-negative colonies on SMAC agar can be tested using *E. coli* O157:H7 antiserum or Latex reagents (O157 antibody-coated latex and control latex) (March and Ratnam, 1989). These recommendations mainly have to do with materials to use and the need to use the test and control latex for each isolate. This is because of cross-reactions with strains of some other bacteria such as *Citrobacter freundi* strain, which carries the *E. coli* O157 antigen (Corbel, 1985; Lior and Borczyk, 1987; Bettelheim *et al.*, 1993). For this reason isolates agglutinating in O157 antiserum or O157 latex reagent should be identified biochemically as *E. coli*. Specimens from which sorbitol-negative colonies

have been isolated that agglutinate in O157 antiserum or O157 latex reagent, and are biochemically *E. coli* may be reported as presumptively positive for *E. coli* O157. Flagella (H7) serology is used for confirmation of *E. coli* O157:H7 as it identifies the flagellar antigen. This is usually performed in reference laboratories. However, it can be ran in clinical laboratories using latex reagents (H7 antibody coated test and control latex). Growing the organism in a suitable broth medium overnight ensures enough flagella antigen for detection (Wang *et al*., 2002).

Toxin testing where H7 serology has been done is not required in outbreak situations because almost all strains of *E. coli* O157:H7 are pathogenic. It could be a requirement for research because some strains of *E. coli* O157:H7 are not pathogenic. Islam *et al*. (2005) used *E. coli* O157:H7 strain B6914, which lacked the *stx1* and *stx2* genes for his experiments. Toxin testing can also be carried out by a reverse passive latex agglutination protocol using commercially available kits (Smith *et al.*, 2003), and by the Vero and HeLa cell culture assays (Blanco *et al*., 1996; Mora *et al*., 2005).

2.5.9 Immunomagnetic Separation

Immunomagnetic separation is based on concentrating the *E. coli* O157 to be isolated by using paramagnetic beads coated with antibodies against *E. coli* O157 antigen in a suspension with the sample usually containing low numbers of the organism. The process is fastened by incubation, constant mixing and pre-enrichment of the suspension. Beads with bound bacteria are separated from the supernatant by a magnetic field and plated on selective media (Wright *et al*, 1994).

The IMS allowed the detection of O157 strains at the 10^2 CFU/g of stool in the presence of 10^7 coliform background flora. A few *E. coli* O157:H7 strains ferment sorbitol (Sernowski and Ingham, 1992; Ojeda *et al.*, 1995), and appear pinkish on SMAC agar. These strains are extremely susceptible to tellurite. Therefore, the sensitivity of culturing can be improved by the use of immunomagnetic separation (IMS) before culturing on cefixime-tellurite SMAC agar (CT-SMAC) (Karch *et al.*, 1996). The major advantage of IMS is its serotype specificity irrespective of phenotypic characteristics. IMS followed by culture on cefixime –tellurite SMAC (CT-SMAC) agar was reported to be twice more sensitive than colony hybridization (Karch *et al.*, 1996) and 100 times more sensitive than direct culture (Chapman *et al* 1994). Commercial systems are available for manual or automated separation (Chapman and Cudjoe, 2001).

2.5.10 Detection of Free Faecal Verotoxins

Detection of free faecal verotoxin by vero-cell assay has been used as a diagnostic tool (Chapman *et al*., 1997; Karch *et al*., 1996). However, this method will detect verotoxin from any VTEC and routine use of cell lines has a drawback of microbial contamination of the cell monolayers and interference with the assay by a number of microbial toxins, which are also active against cultured vero-cells (Karch *et al*., 1996). Detection of free faecal verotoxin can be carried out by enzyme immunoassays (Chapman *et al.*, 1997). These methods are more rapid and less subjective than all culture assays but they lack sensitivity and will detect all VTEC (Karch *et al.*, 1996).

2.5.11 Enzyme immunoassays

These target the O157 antigen and have been described as methods for the detection of the organism in enriched samples from food and the environment (Doyle and Schoeni, 1987). These methods are sensitive but time consuming, technically demanding, expensive, and non-specific (Sernowski *et al.*, 1992).

2.5.12 Molecular tests for E.coli O157:H7

Genetic engineering is a powerful novel technology that has revolutionized the study of all organisms. The genome sequence of man (IHGMC, 2001) and many other living organisms (**http://www.ebi.ac.uk/Genome Rewiews/**) have been reported including *E. coli* O157:H7 genome sequence (Perna *et al.*, 2001). Several nucleic acid based technologies are available for the study of *E.coli* O157: H7.

2.5.12.1 Sequencing

Sequencing is the use of Maxam–Gilbert or Sanger methods (Maxam and Gilbert, 1977; Sanger *et al.*, 1977), to accurately establish the order of the four bases along given DNA fragments. It has led to the elucidation of the genome sequence of *Escherichia coli* O157:H7 (Perna *et al.*, 2001) and the characterization of integron-mediated antibody resistance among shiga-like toxin – producing *E. coli* O157:H7 (Zhao *et al.*, 2001). Zhao *et al.* (2001) used sequencing in conjugation experiments involving *E. coli* O157:H7 strain CVM990 that possessed integrons as donor strains and *E. coli* O157:H7 strain JM263 and *Hafnia alvei* strains CVM1202, CVM1203 and CVM1208 as recipient strains. Class 1 integrons were detected among transconjugants of the recipient strains.

DNA sequencing of the integrons amplified from both the donors and the recipients identified identical *aadA* gene, which was originally present only in the donors.

Sequencing has a fundamental role in molecular methods that are used in the study of *E. coli* O157:H7. Zhao *et al.* (2001) used sequencing in conjunction with PCR. Dillon *et al.* (2005) used it together with a mutiplex PCR for screening of integrons in bacterial lysates. Rashid *et al.* (2006) used sequencing with real-time PCR and Dowd and Ishizaki (2006) used sequencing with microarray technology to study the lineages of two *E. coli* O157:H7 strains.

2.5.12.2 Polymerase Chain Reaction (PCR)

This is a technigue that exponentially amplifies almost any region of a selected DNA molecule. The assay reguires DNA nucleotides (dNTPs), template DNA, primers (oligonucleotides 17-50 nucleotides long, complemtary to a short region on either side of the DNA template) and a heat stable enzyme that catalyzes DNA synthesis, usually, Taq Polemerase. The primers hybridize to the opposite strands of the DNA after it has been denatured. Synthesis of new DNA strands is initiated at 90-95^0 C. The newly synthesized DNA strands detached from the template DNA as the reaction mixture is cooled. A second circle is then initiated. The process is repeated over and over until the desired level of amplification of the target DNA is achieved (Primrose *et al.,* 2003). Several PCRs have been described for identification and characterization of *E. coli* O157:H7 using a number of virulence determinants. The primer sets for amplification of O157:H7 genes often include Stx1, Stx2 UidA, Flic, RfbE, EaeA and EHEC Hly (Cebula *et al.*,

1995; Fratamico *et al.*, 1995; Gannon *et al.*, 1997; Germani *et al.*, 1997; Paton and Paton, 1998; Al-Gallas *et al.*, 2002; Bouvet *et al.*, 2002; Keen *et al.*, 2006).

2.5.12.3 Multiplex and Duplex-PCR

These use two or more primer sets to detect different genes simultaneously. Wang *et al.* (2002) has described a multiplex-PCR detecting the genes encoding the major virulence factors and the genes defining the O157:H7 serotype. The virulence genes detected were Stx_1, Stx_2 Stx_{2c}, Stx_{2d} Stx_{2e}, Stx_{2f}, EHEC *hlyA* and *eaeA* and the two genes $rfbE_{O157}$ and $fliC_{H7}$ that provide genotypic identification of the O157:H7 serotype.

2.5.12.4 Pulsed-Field Gel electrophoresis (PFGE)

This molecular technique is used to study genetic relatedness of isolates by digesting DNA with a suitable restriction endonuclease to generate a set of fragments that are analysed by electrophoresis using agarose gel. The PFGE patterns are used in this way for DNA finger printing i.e. each set of DNA fragments has a definite pattern, described as restriction fragment length polymorphism (RFLP). Patterns can be analyzed manually or by software. The PFGE patterns are considered to be clonally related if they have a similarity coefficient higher than 80% (Bidet *et al.*, 2005). Restriction fragment length polymorphisms (RFLP) of genomic –DNA (Genomic DNA fingerprinting) has been used by Zhao *et al.* (1995) to study shedding of *E. coli* O157:H7 in a survey of dairy herds. Bouvet *et al.* (2002) also carried out restriction digest and agarose electophoresis to study *E. coli* O157:H7 in Frech pork. Interpreting dendogram (chromosomal DNA

restriction endonuclease patterns) produced by PFGE has been used for strain typing (Tenover *et al.*, 1995).

2.5.12.5 Probes

Probes are short single stranded segments of DNA that are complementary to the sequence of interest. Probes are usually tagged with mildly radioactive nucleotides or nucleotides that are linked to a substance that emits light when exposed to certain chemicals (Avers, 1986) for ease of detection of the target sequence. Tenover *et al.*, (1995) and Bidet *et al.*, (2005) used digoxigenin labeled PCR products of stx_2 and *eaeA* genes as probes. Probes are used extensively in autoradiography (DNA Probing) and Southern Blotting method (Avers, 1986).

2.5.12.6 Real-Time PCR

PCR has its application in the detection of genes in organisms by the use of primers that anneal to complimentary segments of the targeted nucleic acid sequence of the organism. Genes may often not be expressed by organisms harbouring them (Rashid *et al.*, 2006) *Escherichia coli* O157:H7 is a commensal organism in cattle, but it is a pathogen in humans. This differential expression of virulence suggests that specific virulence factors are regulated differently in humans and bovine host. Real-time PCR was used to test this hypothesis by relating the expression of several putative virulence genes of *E. coli* O157:H7 (*eae, espA, stx2, rfbE, ehxA* and *iha*) to that of the "housekeeping" gene *gnd* during natural human and experimental bovine infection with *E. coli* O157:H7 (Rashid *et al.*, 2006). Sharma, (2006) also targeted the real-time expression of *rfbE* and *eae* genes of

E. coli O157:H7 by Real-time reverse transcription-multiplex PCR for simultaneous and specific detection of *rfbE* and *eae* genes of *E. coli* O157:H7.

2.5.12.7 Microarray Technology

Micorarray analysis is a rapid, specific and sensitive method for detecting microorganisms with DNA microchips (Jin *et al.,* 2005). Specific oligonucleotide probes of bacterial antigenic and virulence genes of *E. coli* O157:H7 and other related pathogen genes were pre-synthesized and immobilized on a solid support to make microchips. Four *E. coli* O157:H7 specific genes, encoding O157 somatic antigen (*rfbE*), H7 flagellar antigen (*fliC*) and toxins (*stx1, stx2*) were monitored by multiplex PCR with four pairs of specific primers. Fluorescence-Cy3 labeled samples for hybridization were generated by PCR with Cy3-labeled primers. Hybridization was performed under stated conditions. Microchip images were taken using a conifocal fluorescent scanner. Twelve different strains were detected with various combinations of four virulent genes. All O157:H7 strains yielded positive results by multiplex PCR. All the probes specifically recognized Cy-3 labeled fluorescent samples from O157:H7 strains or strains containing O157 and H7 genes. No cross hybridization of O157:H7 samples occurred in the probes.

Non-O157:H7 pathogens failed to yield any signal under comparable conditions. If Cy3-labeled fluorescent product of O157 single PCR was diluted 50-fold, no signal was found in agarose gel electrophoresis, but a positive signal was found in microarray hybridization. Jin *et al.* (2005) concluded that Microarray analysis of O157:H7 is a rapid, specific and efficient method for identification of bacterial pathogens. Dowd and Ishizaki

(2006) used a 610 gene microarray designed against the *E. coli* O157:H7 transcriptome to study differential gene expression between two *E. coli* O157:H7 lineages. Their result highlights indicated that under in vitro anaerobic growth conditions, there is an up-regulation of *stx2, ureD, wrli* and stress related genes *hs1J, aspG ibpB, ibpA* which may contribute to enhance virulence or transmission potential. The result gives an insight into comparative regulation of virulence genes as well as providing direction for future research.

Microarray technology has been employed for rapid DNA serotyping of *Escherichia coli* by selecting the genes encoding the O and H antigens to produce probes. This method is much faster and more specific than conventional serotyping (Ballmer *et al.*, 2007).

2.5.12.8 Whole Genome PCR Scanning (WGP Scanning)

Ogura *et al.* (2006) used WGP scanning in combination with the use of the O157 Sakai Oligo DNA Microarray. They concluded that the combined use of the two methods provided much further details on the O157 genome diversity than illustrated by each method alone. WGP scanning involves prior sequencing of the genome of the organism (Ogura *et al.*, 2006) Genome sequencing revealed that most of the large O157-specific sequences are prophages and prophage-like elements, which have carried many virulence genes into the O157 genome. This suggests that bacteriophages have played a key role in the emergence of O157. WGP scanning has revealed a high level of diversity in O157. WGP scanning can be used to monitor acquisition of new genes such as virulence determinants or antimicrobial resistance (Ogura *et al.*, 2006).

2.6 Transmission and Susceptibility to *E. coli* O157:H7 Infection

Escherichia coli O157:H7 infections were initially reported to have been acquired through the consumption of fresh unpasteurized milk, ground beef, hamburgers and sausages (Riley., *et al* 1983; Levine., 1987; Karmali., 1989; Griffin and Tauxe., 1991). A growing list of foods have been associated directly or indirectly with outbreaks of the infection in many parts of the world, including unpasteurized apple cider in the USA (Besser *et al.*, 1993), apple juice in British Columbia, California, Colorado and Washington, USA (USDHHS/CDC, 1996), salad and vegetables in Egypt (Abdoul-Raouf *et al.*, 1996), dry cured salami, in Washington State USA (Alexander., *et al.* 1995), raw milk in Oregon, USA (Armstrong *et al.*, 1996), water (recreational and table water) in Missouri, USA, Swaziland, southern Africa, UK, Japan and Kenya (Swerdlow *et al.*, 1992; Sang *et al.*, 1996; Izumiya *et al.*, 1997; Morgan *et al.*, 1998; Effler *et al.*, 2001), meat and sausages in Italy (Bonardi *et al.*, 1999; Normanno *et al.*, 2004). Manure was implicated in the faecal-oral route (Cieslak, 1993). It is now commonly accepted that most *E. coli* O157:H7 associated diseases of humans have a bovine origin: *E. coli* O157:H7 colonizes the digestive tract of cattle with no clinical signs (Montenegro *et al.*, 1990; Garber *et al.*, 1995), and is transmitted to man by contamination of food and water with faeces from infected animals during periods of transient shedding of the organism in their faeces (Garber *et al.*, 1995). Contamination of carcasses can occur at the abattoir (even when the animals are infected but not shedding the organism) and during processing (Li *et al.*, 2004). Person-to-person transmission and occupational exposure have been reported (Armstrong *et al.*, 1996).

Children five years or less in age are reported to be more susceptible to *E. coli* O157:H7 infection (Effler *et al.*, 2001). Female gender seemed to be a risk factor in the outbreak in Swaziland and South Africa (Effler *et al.*, 2001). *E. coli* O157:H7 has been reported as the sole cause of HUS in North America. The syndrome is mainly a disease of children (http://www.about-ecoli.com/main.html), however, *E. coli* O157:H7 has been reported in all ages in man. The elderly appear to be second in susceptibility as an age bracket accounting for most cases of TTP. Effler *et al.* (2001) reported a five-fold increase in cattle death during the outbreak in Swaziland but specific work needs to be done to attribute death to the pathogen in infected cattle.

2.7 Reservoir and Occurrence of *E. coli* O157:H7

Epidemiological investigations have revealed that dairy cattle especially young animals are the principal reservoir of *E. coli* O157:H7 (Borczyk *et al.*, 1987; Montenegro *et al.*, 1990; Chapman *et al.*, 1993b; Hancock *et al.*, 1994; Zhao *et al.*, 1995). The organism colonizes and multiplies in the digestive tract of these animals with the animal being clinically healthy (Montenegro *et al.*, 1990; Garber *et al.*, 1995). Faecal shedding occurs only transiently and the animal continues to harbor the infection even when faecal shedding has ceased, (Mechie *et al.*, 1997; Paiba *et al.*, 2003).

Studies have revealed that *E. coli* O157:H7 is more frequently carried and shed by calves and heifers than by adult cattle (Orskov *et al.*, 1987; Wells *et al.*, 1991; Wilson *et al.*, 1993;Garber *et al.*, 1995). These investigations came since the first two outbreaks of *E. coli* O157:H7 infection, which were associated with undercooked ground beef after which cattle became suspected as an important source of the pathogen (Martin *et al.*,

1986; Borczyk *et al.*, 1987; Orskov *et al.*, 1987 Clarke *et al.*, 1989; Montenegro *et al.*, 1990; Chapman *et al.*, 1993a). The carriage by animals indicates that beef cattle other than dairy cattle are the next important reservoir of *E. coli* O157:H7 followed by sheep (Paiba *et al.* 2000). *E. coli* O157:H7 has also been isolated from pigs, deer, bison, goats, poultry, wild birds faeces, rabbits and flies (Chapman *et al.*, 1997; Pritchard *et al.*, 2001; Fischer *et al.*, 2001; Rice *et al.*, 2003), but it is generally accepted that cattle with a higher prevalence rate (Chapman *et al.*, 1997), higher carriage than sheep and pigs (Paiba *et al.*, 2000) are the primary reservoir species. More so in dairy herds which show higher herd prevalence. Calves and heifers within dairy herds show up to 10^5 CFU/g of faces of *E. coli* O157:H7 while shedding and showing no clinical signs of infection for up to an upper limit of a year (Zhao *et al*, 1995).

2.8 Prevalence and Infective Dose of *E. coli* O157:H7

The methods used for isolating *E. coli* O157:H7 greatly influence the isolation rate (Armstrong *et al.*, 1996). Direct plating of faecal specimens without an enrichment procedure was less sensitive. Sensitivity was improved by enrichment of the samples. The minimum level of sensitivity of direct plating procedure is 10^2 CFU/g of faeces (Zhao *et al.*, 1995). The ELISA procedure used by Zhao *et al.* (1995) enabled them to focus on specific samples and to test, if necessary all colonies typical of *E. coli* O157:H7 on sorbitol negative and MUG negative (SMA-MUG) plates. Where better detection procedures are used, greater prevalence of *E. coli* is likely to be identified (Doyle, 1991, Padhye and Doyle, 1991).

Studies of cattle in the North America and Europe have generally reported prevalence of *E. coli* O157:H7 as ranging from less than 1 to 16% (Chapman, 1997; Garber *et al.*, 1995; Martin *et al.*, 1986; Orskov *et al.*, 1987; *Wells et al.*, 1991), with levels as high as 36% in a few selected studies (Armstrong *et al.*, 1996).

The upper limits were reported from dairy herds in calves and heifers (Zhao *et al.*, 1995). Herd prevalence rates are high in the USA. Zhao *et al.* (1995) reported that 50% of the case herds they sampled showed at least one positive sample. Higher prevalence in dairy herds (Hancock *et al.*, 1997b) have been reported when compared to beef herds (Hancock *et al.*, 1997a) in the same environment.

Prevalence in other animals has been reported. Bouvet *et al.* (2002) reported a figure of 0.6% (3/498) using a PCR to screen carcasses and pork in France. Rice *et al.* (2003) reported prevalence of 0.79%, 0.34% and 3.33% in white-tailed deer, wild bird feces and flies respectively. Li *et al.* (2004) reported a figure of 1.4% on Bison carcasses in the USA.

Escherichia coli O157:H7 has been recovered from cattle faeces and the rumen (Van Donkersgoed *et al.*, 1999), hide (Elder *et al.*, 2000) and numerous studies have reported the presence of the organism on retail meats (Johnson *et al.*, 1998, Normano *et al.*, 2004, Vernozy-Rozand *et al.*, 2002, Walsh., 1997, Willshaw *et al.*, 1994), usually at a low prevalence. Vernozy-Rozand *et al.* (2002) reported 0.12% (4/3450) samples positive for *E. coli* O157:H7 in large scale processed minced meat in France. Studies of butcher shops in a range of countries have reported higher values: Chinen *et al.* (2001) in Argentina

2.8% (6/160), Chapman *et al.* (2000) in the UK, 1.1% (36/3216). In the Netherlands, Heuvelink *et al.* (1999) reported an overall prevalence of 1.1 (6/571) of *E. coli* O157:H7 in minced beef products.

In a study of beef burgers and in minced beef quantification, the most probable number (MPN) technique was used and one sample was reported to contain 2300 $cfug^{-1}$ (3.66 $log_{10}cfug^{-1}$) (Bolton *et al.*, 1996).

Prevalence of *E. coli* O157:H7 in various domestic animals and flies were reported recently by Keen *et al.* (2006). It has been reported that high prevalence (up to 60%), have been observed when cattle are together with infected persons (Armstrong *et al.*, 1996). Synge and Hopkins (1996) reported a similar observation on a farm in Scotland.

Since an outbreak study reported by Willshaw *et al.* (1994), where contamination levels in an implicated product were reportedly as low as 2 cells per 25g, infective doses for *E. coli* O157:H7 have been reported by many workers and the range is 2-10 cells (Coia, 1998; Doyle *et al.*, 1997). Since then, isolation of *E. coli* O157:H7 has been reported with no attempts to quantify. Emphasis is being shifted to survival times of *E. coli* O157:H7 on various items (Islam *et al.*, 2005), including foods and soil.

2.9 Control

The control of VTEC is an integral part of its prevention from reaching man. Hence it is centered on the animals, farm, abattoir and the environment. This strategy ensures that VTEC does not enter the food chain and access factors contributing to the emergence and survival of VTEC in animals and the environment (Armstrong *et al.*, 1996; Effler *et al.*, 2001).

Vali *et al.* (2005) reported that different VTEC strains are shed by the same cattle under different environmental conditions and diets which affect the bovine gastrointestinal ecology and suggested that populations of VTEC especially *E. coli* O157:H7 are responsive to feeding and management practices, which could be exploited to develop strategies for controlling VTEC in general and *E. coli* O157:H7 shedding by cattle in particular. Several studies have reported on the effect of the environment on the *E. coli* O157:H7 in cattle. Suggestions have also been made that the acidity in grain diets makes *E. coli* O157:H7 that have been fed on grains or special diets to be more pathogenic to man. The acid level in the animal's gastrointestinal tract act to induce acid tolerance by the expression of the *rpoS* gene. Sharma and Beuchat (2004) reported that *E. coli* cells deficient in the expression of the *rpoS* gene were more susceptible to acidic, osmotic and heat stress than wild-type cells.

2.9.1 *Bactriophage Therapy*

Bacteriophages (phages) are bacterial viruses that specifically target bacteria. Each phage is specific for only one species and in some cases only a single strain of bacterium (Hanlon, 2007). The use of bacteriophage as a therapeutic agent is a possible option for controlling pathogenic bacteria (Bohannan and Lenski, 1997, Levine *et al.*, 1997). Phage therapy was developed early in the last century and showed much promise, but aroused much controversy. Extensive clinical research and implementation of phage therapy has continued to be carried out in Easten Europe over the last 50 years (Alisky *et al.*, 1998). The recent increasing emergence of multiantibiotic-resistant mutants of pathogenic bacteria and the decline in the rate of drug development revived phage therapy in Western countries (Angel *et al.*, 1987; Powers, 2004).

Therapeutic use of phage cocktail for controlling *E. coli* O157:H7 is being studied *in vitro* and *in vivo* in mice and it offers promise (Tanji *et al.*, 2005). Repeated oral administration of $SP_{15\text{-}21\text{-}22}$ phage cocktail was effective for rapid evacuation of *E. coli* O157:H7 from the faeces and gastrointestinal tract of animals and animal models of human infection (Barrow, 2001).

2.9.2 Probiotics

Probiotics also referred to as competitive exclusion of products (CEP) are agents or products that are used to create an unfavourable environment for a pathogen and therefore reduce its survival within a host. This could be by the use of probiotic bacteria (competitive – exclusion bacteria) (Brashears *et al.*, 2003; Tkalcic *et al.*, 2003) or probiotic substances such as probiotic sugars to displace the targeted organism within the host (De Vaux *et al.*, 2002). When inoculated into raw rumen broth containing 3g of sorbitol per liter, *E. coli* O157:H7 was displaced within 72 hours. Similar results were obtained by addition of other competitive sugars, such as L-arabinose, trehalose, and rhamnose. The result agreed with the therapy that a robust population of commensal ruminal microflora is required to involve competitive exclusion of *E. coli* O157:H7 by the addition of sugars that *E. coli* O157:H7 cannot ferment. This approach may be effective as a preharvest strategy for reducing carriage of *E. coli* O157:H7 in the rumen and thus lower its chances of entering the food chain of humans (De Vaux *et al.*, 2002). Other substances such as volatile fatty acids have been used (Wolin, 1969). Commercial competitive exclusion products are available for controlling of *E. coli* O157:H7 shedding in cattle (Brashears *et al.*, 2003).

2.10 Situation of *E. coli* O157:H7 in Africa

2.10.1 West Africa

In Nigeria the early works on enteroheorrhagic *E. coli* (EHEC) and Shiga toxin-producing *E. coli* (STEC) were carried out by Ogunsanya *et al.* (1989) who reported a 5.1% prevalence of EHEC infections among paeditric patients with diarrhoea in Lagos. Tekdek (1994) reported isolation frequencies of 3.3% and 9.3% from diarrhoeic and non-diarrhoeic calves in Zaria. Olorunshola *et al.* (2000) later reported a prevalence rate of 6% of patients with EHEC O157:H7 in Lagos. They observed that the situation had remained the same since the earlier report by Ogunsanya and co-workers in 1989. Akinyemi *et al.* (1998) studied *E. coli* O157:H7 infections in Lagos and reported that it occurred in all age groups studied. Okeke *et al.* (2000) in a study in small towns and rural primary health centers in South-western Nigeria reported characterization of EHEC *E. coli*. An overview of the reports from Nigeria indicate that children were the most affected age group (Akinyemi *et al*., 1998; Olorunshola *et al*., 2000), and all the isolates were susceptible to most of the antimicrobials tested which included gentamicin, streptomycin, nitrofurantoin, nalidixic acid, ampicillin, sulphamethoxazole, co-trimoxazole and tetracycline. All the patients from whom *E. coli* O157:H7 were isolated had gastroenteritis and HC. Resistance was reported to suphametoxazole, co-trimoxazole and tetracycline (Olorunshola, *et al.*, 2000), but no resistance patterns were reported. The only studies in animals seem to be that by Smith *et al.* (2003) and Tekdek (1994). Smith *et al.* (2003) reported a presumptive identification of *E. coli* O157:H7 by a latex agglutination test from various animals around Lagos, Nigeria. Tekdek (1994) carried out

epidemiologic and bacteriologic studies of calf collibacillosis in herds of cattle in Zaria, Northwestern Nigeria and reported the occurrence of numerous serogroups in calves.

Dadie *et al.* (2000) was the first report of *E. coli* O157:H7 infection in Cote d'Ivoire. The isolate was from a child and harbored the *stx2*, *eae* and *uidA* genes but did not posses the EHEC *hlyA* gene.

2.10.2 North Africa

The only report available from North Africa is from Abdoul-Raouf *et al.* (1996), who sampled some Egyptian foods. Their survey was done in middle Egypt to determine if *E. coli* O157:H7 was present in 175 samples of raw ground beef, chicken, lamb and unpasteurized milk. The pathogen was detected in 3 of 50(6%) beef samples, 2 of 50 (4%) chicken samples, 1 of 25 (4%) lamb samples and 3 of 50 (6%) milk samples obtained from slaughter houses, supermarkets and farmer's homes.

2.10.3 Central Africa

Escherichia coli O157:H7 infections have been reported in Cameroon in Central Africa (Germani *et al.*, 1997, 1998; Cunin *et al.*, 1999,). In outbreaks of bloody diarrhea *Shigella dysenteriae 1*, *Shigella boydii* and EHEC *E. coli* O157:H7 were isolated. All the pathogens were multidrug resistant (MDR) strains.

2.10.4 *East Africa*

Hemorrhagic colitis (HC) due to EHEC O157:H7 has been reported in Kenya (Sang *et al.*, 1996).

2.10.5 *Southern Africa*

Escherichia coli O157:H7 infection was first reported in South Africa in March, 1990 by Browning *et al.* (1990). A large outbreak of HC was reported in Swaziland, southern Africa in 1992 by Effler *et al*. (2001). The outbreak was reported to have spread into South Africa (Effler *et al.*, 2001). In Swaziland, 40912 physician visits for diarrhea in persons ages $\geq$ 5years were reported during October through November 1992. The attack rate was 42%. Female gender and consuming beef and untreated water were significant risks for illness. *E. coli* O157:H7 was recovered from seven affected foci in Swaziland and South Africa. The isolates had indistinguishable pulsed-field gel electrophoresis patterns. The first heavy rains, drought and carriage of *E. coli* O157:H7 by cattle were reported as important risk factors contributing to the outbreak. Muller *et al.* (2001) screened South African water sources intended for direct and indirect human consumption using the chromogenic rainbow agar O157 medium and reported that none of the colonies agglutinated with *E. coli* O157:H7 antisera. Effler *et al*. (2001) reported that by 1993, no *E. coli* O157:H7 was detected in patient stools or environmental specimens obtained from the affected areas in Swaziland and South Africa. Effler *et al.* (2001) concluded from their overview of the situation in Africa and concluded that the pathogen has wide geographic distribution in Africa.

2.10 Antimicrobial Resistance: an Overview

Since the dawn of the antimicrobial drug era, resistance has cast a shadow on the success of infectious disease therapy. Alexander Fleming warned about the danger posed by resistance as early as the 19th century in his Nobel Prize acceptance speech; “It is not

difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them… if you use ”penicillin, use enough”. Available at: **http://nobelprize.org/medicine/laureates/1945/fleming-lecture.pdf2**.

In this dawn of the twenty-first century our understanding of resistance has grown vastly more sophiscated. The molecular basis for antimicrobial resistance has been elucidated for many microbes. (Halter *et al.*, 1989; Kell *et al.*, 1993; Hakenbeck, 1995; Drlica and Zhao, 1997; White *et al.*, 2001; Zhao *et al.*, 2001;Dillon *et al.*, 2005; Hopkins *et al.*, 2005) but the proliferation of new antimicrobial drugs has engendered an equally varied collection of resistance mechanisms.

2.11.1 *Resistance to Sulfonamides*

The sulfonamides were among the first antimicrobial compounds to be widely used in the treatment of disease. They are substrate analogues that act as competitive inhibitors of dihydropterate synthase, encoded by the *dhps* gene, which catalyzes the formation of dihydropteric acid as part of the folate synthesis pathway. All bacteria have this pathway but is not present in mammals. Therefore sulfonamide drugs do not affect human metabolism. Import of complete chromosomal *dhps* genes and *dhps* mosaic alleles from a different species has given rise to sulfonamide resistance (Kristiansen *et al.*, 1990; Fenner *et al.*, 1995; Radstrom *et al.*, 1992). This import is through horizontal genetic exchange between bacterial (Maynard *et al.*, 1991; Maiden, 1993, 1998).

2.11.2 *Resistance to β-Lactam Antibiotics*

β-lactam antibiotics are among the most commonly used antimicrobial agents. They act on penicillin binding proteins (PBPs), which are involved in cell wall synthesis. Penicillin, a β-lactam antibiotic, was one of the first antibiotics. B-Lactam antibiotics are still the most widely used and diverse class of drugs used clinally, and new members are still being developed. Resistance to many β-lactam compounds is commonplace and still evolving. Resistance is most often caused by the presence of β-lactamases, but mutations in PBPs resulting in reduced affinity for β-lactam antibiotics are also commonly observed. Resistance is less frequently caused by reduced uptake due to changes in the cell wall or active efflux (Fluit *et al.*, 2001).

Several classification systems for β-lactamases space have been published. One classification scheme is based on their nucleotide sequence, classes A through D (Bush *et al.*, 1995). Class A, C, and D enzymes have a serine at their active site, while class B enzymes have four zinc atoms at their active site. Class A enzymes are highly active against benzylpenicillin. The extended-spectrum β-lactamases (ESBLs) belong to class A enzymes. Extended-spectrum β- lactamases (ESBLs) inactivate benzylpenicillins as well as some cephalosporins and/or monobactams. Class B β-lactamases are equally active against penicillins and cephalosporins, and at least some of these enzymes are able to inactivate carbapenems. Class C genes are usually inducible, but mutations can lead to overexpression. Class D is composed of the OXA-type enzymes, which are capable of hydrolyzing oxacillin. Genes encoding β-lactamases can be located either on plasmids or

the bacterial chromosome and are found among both gram-negative and gram-positive organisms (Fluit *et al.,* 2001; Branger *et al.*, 2005; Lavingne *et al.*, 2007).

2.11.3 *Resistance to Aminoglycosides*

Aminoglycosides such as gentamicin, tobramycin, amikacin, and streptomycin are commomly used antimicrobial agents in the treatment of infections by both gram-negative and gram positive organisms. Aminoglycosides bind to the ribosomes and thus interfere with protein synthesis. Resistance to these antimicrobial agents is widespread, with more than 50 aminoglycoside-modifying enzymes already described. Most of these genes are associated with gram-negative bacteria. Depending on their type of modification, these enzymes are classified as aminoglycoside acetyltransferases (AAC), aminoglycoside adenylyltransferases (also named aminoglycoside nucleotidyltransferases [ANT]), and aminoglycoside phosphotransferases (APH) (Fluit *et al.,* 2001). Besides aminoglycoside-modifying enzymes, efflux systems and rRNA mutations have been described (Shaw *et al* 1993; Quintiliani and Courvalin , 1995).

2.11.4 *Resistance to Chloramphenicol*

Chloramphenicol binds to the 50S ribosomal subunit and inhibits the peptidyltransferase step in protein synthesis. Resistance to chloramphenicol is generally due to inactivation of the antibiotic by a chloramphenicol acetyltransferase. The *cat* genes of gram-negative and gram-positive bacteria show little homology, and a variety of different enzymes have been described. The gene is found on plasmids in *E. coli.* Sometimes decreased outer

membrane permeability or active efflux is observed in gram-negative bacteria (Schmitz and Fluit, 1999).

2.11.5 *Resistance to Tetracyclines*

Tetracyclines probably penetrate bacterial cells by passive diffusion. Tetracycline acts by binding to the 30S ribosomal subunit, resulting in the inhibition of protein synthesis (Chopra *et al.*, 1992; Klein and Cunha, 1995; Schnappinger and Hillen 1996). A growing number of bacterial species have acquired resistance to the bacteriostatic activity of tetracycline. In 1989, a note describing the nomenclature for tetracycline resistance determinants which employed letters of the English alphabet was published by Levy *et al.* (1989). Most of the resistance genes code for one of the two important mechanisms of tetracycline resistance, efflux or ribosomal protection. These two widespread mechanisms of bacterial resistance to tetracycline do not destroy the compound. Efflux is mediated by energy-dependent efflux pumps; the other important mechanism involves an elongation factor G like protein that confers ribosome protection.

Oxidative destruction of tetracycline has been found in a few species. Nevertheless, the enzymatic inactivation of the antibiotic is not thought to be important in nature (Levy *et al.*, 1989; Levy 1992a; Levy 1992b; Carbello *et al.*, 1994; Roberts, 1994; Roberts, 1996; Levy *et al.*, 1999; Schnappinger and Hillen, 1996; Taylor and Chau, 1996). An overview of the distribution of the better-studied tetracycline resistance determinants among gram-negative and gram-positive genera has been given in reviews by Roberts (Roberts, 1994; Roberts, 1996). The efflux proteins exchange a proton for a tetracyclinecation complex

and are antiporter systems. Efflux determinants from gram-negative bacteria (TetA to TetE, TetG, and TetH) have a common genetic organization that is different from that in gram-positive bacteria. They all contain a structural gene and a repressor gene that are expressed in opposite directions from overlapping operator regions. The gram-positive *tetK* and *tetL* genes encoding tetracycline efflux proteins are regulated by mRNA attenuation in a similar way to that described for gram-positive *erm* genes encoding rRNA methylase and *cat* genes encoding chloramphenical acetyltransferases (Levy, 1988; Levy, 1992a; Levy 1992b; Roberts 1994; Roberts 1996; Taylor and Chau, 1996) Schannpinger and Hillen 1996; Levy *et al.*, 1998; Levy *et al*, 1999). The efflux proteins confer resistance to tetracycline but not to minocycline. Protection of the ribosome from the action of tetracycline as a mechanism of tetracycline resistance was discovered in streptococci. Tetracycline resistance can result from the production of a protein that interacts with the ribosome such that protein synthesis is unaffected by the presence of the antibiotic. The determinants TetM, TetO, TetB(P), TetQ, TetS, TetT, TetW, and OtrA confer resistance to tetracycline, doxycycline, and minocycline (Levy, 1988; Levy, 1992a; Levy 1992b; Levy *et al.*, 1998; Levy *et al*, 1999). The *tetX* gene codes for an enzyme which inactivates tetracyclines.

This enzyme is a novelty because it is the first and only enzyme described which inactivates tetracyline (Taylor and Chau, 1996;Levy *et al.*, 1999;). Neither the prevalence nor the clinical significance has been studied so far. According to data from Roberts (Roberts, 1994; Roberts, 1996), specific tetracycline resistance genes have been identified in at least 32 gram-negative including *E. coli*.

2.11.6 *Resistance to Quinolones (Including Fluoroquinolones)*

Quinolones and their fluorinated derivativesexert their antibacterial effects by inhibition of certain bacterial topoisomerase enzymes, namely, DNA gyrase (bacterial topoisomerase II) and topoisomerase IV. These essential bacterial enzymes alter the topology of double-stranded DNA (dsDNA) within the cell (Quintiliani and Courvalin, 1995; Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1998).

DNA gyrase and topoisomerase IV are heterotetrameric proteins composed of two subunits, designated A and B. The genes encoding the A and B subunits are referred to as *gyrA* and *gyrB* (DNA gyrase) or *parC* and *parE* (DNA topoisomerase IV in *E. coli* (Quintiliani and Courvalin, 1995; Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1998). DNA gyrase is the only enzyme that can effect supercoiling of DNA.

Inhibition of this activity by all quinolones including the fluoroquinolones is associated with rapid killing of the bacterial cell. Topoisomerase IV also modifies the topology of dsDNA, but while DNA gyrase seems to be important for maintenance of supercoiling, topoisomerase IV is predominantly responsible for the separation of daughter DNA strands during cell division (Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1998; Quintiliani and Courvalin, 1995). Mechanisms of bacterial resistance to quinolones and their fluorinated derivatives fall into two principal categories, alterations in drug target enzymes and alterations that limit the permeation of drug to the target. The target

enzymes are most commonly altered in domains near the enzyme active sites and in some cases reduced drug binding affinity has been demonstrated (Hooper, 1999).

In gram-negative organisms, DNA gyrase seems to be the primary target for all quinolones (Quintiliani and Courvalin, 1995; Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1998). Alterations of target enzymes appear to be the most dominant factors in expression of resistance to quinolones. The small region from codons 67 to 106 of GyrA in *E. coli*, the most extensively studied organism, was designated the quinolone resistance-determining region (QRDR). The QRDR is near Tyr122, which is transiently covalently bound to DNA phosphate groups during the enzyme's DNA strand-passing reactions (Fluit *et al.*, 2001).

In almost all instances, amino acid substitutions within the QRDR involve the replacement of a hydroxyl group with a bulky hydrophobic residue. This suggests that mutations in *gyrA* induce changes in the binding-site conformation and/or charge that may be important for quinolone-DNA gyrase interaction. Although quinolones are thought to interact primarily with the A subunit of DNA gyrase, mutations have also been discovered in the B subunit which also confer quinolone resistance in some species such as *E. coli*. However, the frequency of *gyrB* mutations, compared to *gyrA*, has been shown to be relatively low in most species (Quintiliani and Courvalin, 1995; Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1998).

Topoisomerase IV alterations have now been characterized in several bacteria. The ParC subunit of topoisomerase IV is homologous to GyrA, and the ParE subunit is homologous to GyrB. Particularly highly conserved in the topoisomerase IV homologs are the QRDR domains of GyrA and GyrB, a conservation that predicts similarity of drug interaction and resistance mutations for the two enzymes (Fluit *et al.*, 2001).

DNA gyrase and topoisomerase IV are both located in the cytoplasm of the bacterial cell. To reach their targets, quinolones and fluoroquinolones must traverse the cell envelope. Changes in the cell envelope of gram-negative bacteria, particularly in the outer membrane, have been associated with decreased uptake and increased resistance (Fluit *et al.*, 2001).

2.11.7 *Mechanisms of Resistance to Antimicrobial Agents in E. coli*

Usually resistance arises spontaneously due to point amino acid substitution within the topoisomerase genes. This often is in combination with decreased outer membrane porins and over-expression of multi-drug efflux pumps (Hopkins *et al.*, 2005). This explains why quinolone resistance is often accompanied by resistance to three or four other antimicrobials as was reported by Threlfall *et al.* (1998). Recombination also has an important role in the evolution of antibiotic resistance determinants. Progressive clustering of genes leading to an operon structure could optimize the expression of ancient genes producing a resistant phenotype (Lawrence and Roth, 1996).

Mutations in DNA gyrase of *E. coli:* A single mutation in *gryA* of *E. coli* is sufficient to cause high level resistance to nalidixic (Vila *et al.*, 1994; Ozeki *et al.*, 1997), but additional mutations in *gryA* and/or topoisomerase IV genes occurring in a stepwise manner have been reported for high level fluoroquinolone resistance (Vila *et al.*, 1994; Heisig, 1996; Ozeki *et al.*, 1997).

Nucleotide substitutions at codon 83 are the most common gyrA mutations found in clinical veterinary and laboratory strains of *E coli*. In *E. coli* strains that demonstrated high level resistance to nalidixic acid, this serine residue is most commonly substituted with a leucine (Vila *et al.*, 1994; Ruiz *et al.*, 1995; Everett *et al.*, 1996). An additional mutation, most commonly at codon Asp87, is associated with slightly higher resistanace to fluoroquinolones (Vila *et al.*, 1994; Ozeki *et al.*, 1997; Saenz *et al.*, 2003). However, strains with a single mutation at Ser83 were significantly more resistant to fluoroquinolones than those with a single mutation at Asp87 (Ozeki *et al.*, 1997, Bagel *et al.*, 1999), thus providing a plausible explanation for the higher prevalence of mutations at Ser83 in high resistance strains of *E coli.*

Mutation in Topoisomerase IV gene of *E. coli*: Previous studies have shown topoisomerase is not as sensitive to quinolones as DNA gyrase (Kato *et al.*, 1992; Peng and Marians, 1993), and is therefore, a secondary target for quinolones in *E. coli* and other gram-negative bacteria (Khodursky *et al.*, 1995). Mutations in *parC* and parE of *E. coli* have always been found together with mutations in *gryA* (Everett *et al.*, 1996; Heisig, 1996; Fendukly *et al.*, 2003; Komp *et al.*, 2003; Saenz *et al.*, 2003), suggesting that the

mutations in topoisomerase IV will nut occur unless the sensitivity of the DNA gyrase to fluoroquinolones has been reduced to at least that of topoisomerase IV by DNA gyrase mutations (Heisig, 1996, Everett *et al.*, 1996). A single mutation within *parC* occurring alone in an in vitro mutant did not affect quinolone susceptibility of the resulting strain (Bagel *et al.*, 1999)

Decreased Fluoroquinolone Uptake: High level fluoroquinolone resistance in *E. coli* is usually associated with multiple mutations in the quinolone resistance-determining region (QRDR) of the topoisomerase genes (Heisig *et al.*, 1993). Highly resistant *E. coli* strains with at least two mutations in *gryA* and an additional mutation in *parC* may exhibit the same level of fluoroquinolone resistance as salmonella strains with only *gryA* mutation (Giraud *et al.*, 1999). In addition strains of *E. coli* and salmonella that habour identical mutations in the topoisomerase genes, yet have different Cip MICs have been identified (Conrad *et al*, 1996; Giraud *et al.*, 1999; Linde *et al.*, 2000). Mutations in the topoisomerase genes therefore, cannot totally account for resistance phenotypes of these strains, suggesting additional mechanism(s) of resistance.

Decreased fluoroquinolone uptake can be due to an increase in bacterial impermeability, unavailability of the drug in the immediate environment of the bacteria and/or overexpression of efflux pumps (Hopkins *et al.*, 2005).

Quinolone resistance resulting from the multiple antimicrobial resistance (MAR). Multidrug resistance (MDR) phenotype is often associated with cross-resistance to many other structurally unrelated antimicrobials such as beta-lactams, puromycin, tetracyclines,

and chloramphenicol (George and Levy, 1983), and resistance to oxidative stress agents, disinfectants and to some organic solvents such as cyclohexne (Ariza *et al.*, 1994, Moken *et al.*, 1997, McMurry *et al.*, 1998). Expression of *mar* locus also provides cross-resistance to fluoroquinolones. In *E. coli* this could be a first step towards high level fluoroquinolone resistance by allowing surviving cells to mutate to clinically relevant levels of fluoroquinolone resistance (Goldman *et al.*, 1996).

The mar phenotype in *E. coli:* In *E. coli,* the *mar* operon is located at 34 min on the chromosome (George and Levi, 1983). The *mar* operon consists of two transcriptional units *marC* and *marRAB.* In *E. coli* mutations can occur leading to increased or constitutive expression of the *mar* operon.(Cohen *et al.*, 1993a,b; Maneewannakul and Levy, 1996; Komp *et al.*, 2003).The MAR phenotype is also inducible by tetracyclines, chloramphenicol, salicyclates and many other substances with phenolic rings, Cohen *et al.*, 1993b; Seoane and Levy, 1995).

Plasmid Mediated Quinolone Resistance (PMQR) in *E. coli:* PMQR was first reported in *Shigella dysenteriae* (Munshi *et al.*, 1987). A plasmid that conferred reduced susceptibility to *E. coli* was described by Martinez *et al.* (1998). The gene responsible for PMQR has been identified as *qnr* (Tran and Jacoby, 2002). *Qnr* protect *E. coli* gyrase from inhibition by ciprofloxacin (Hopkins *et al.*, 2005). PMQR is very rare (Jacoby *et al.*, 2003), but they have recently been associated with class 1 integrons and reported in *Klebsiella pneumoniae, Shigella dysenteriae, Escherichia coli* O157:H7 and other STEC serotypes from China to the USA (Munshi *et al.*, 1987; Zhao *et al.*, 2001;

Rodriquez-Martinez *et al.*, 2003; Wang *et al.*, 2003, 2004). The threat is that *E. coli* O157:H7 which has acquired SLT from S*hingella dysenteriae* 1 (that produces ST) through the mediation of phages and is a novel organism and forerunner for gene acquisition (Ogura *et al.* 2006).

2.11.8 *Detection of Resistance*

Various methods have been reported to detect point mutations in target genes, including sequence-specific oligonucleotide probe hybridization (Fashching *et al., 1991*), sequencing of the target genes (Granjeaud *et al.*, 1999), RFLP (Sreedharan *et al.*, 1990; Sreedharan *et al.*, 1991), radioisotopic or nonradioisotopic SSCP analysis (Hayashi, 1991; Sugano *et al.*, 1993; Tokue *et al.*, 1995; Oubdesselam *et al.*, 1995), mismatch amplification mutation assay (MAMA)- PCR, and allele-specific PCR in combination with RFLP (Giraud *et al.*, 1999).

Giraud *et al.* (1999) developed a rapid assay combining allele-specific PCR and RFLP (AS-PCR-RFLP) for the screening of point mutations responsible for all amino acid changes encoded by the *Salmonella enterica gyrA* gene at codons 81, 83 and 87. These alterations were most frequently encountered in fluoroquinolone-resistant clinical than laboratory strains.

2.11.9 *Multidrug Resistance*

Often bacterial isolates are multidrug resistant. However, the vast majority of studies have looked at the detection and diagnosis of resistance caused by one class of ntimicrobial agents while others have looked at the detection of resistance to multiple

antimicrobial agents. Integrons are a special case of multidrug resistance. Integrons are genetic elements that contain the genetic determinants of a site-specific recombination system that recognizes and captures mobile gene cassettes. An integron contains an integrase and an adjacent recombination site. Gene cassettes can be integrated by the integrase at the recombination site, and multiple gene cassettes can be present in one integron (Hall and Collins, 1995). Four classes of integron have been described. Class 1 integrons are often associated with the sulfonamide resistance gene *sulI* and are the most common integrons. Class 2 integrons are associated with Tn*7*. Only one class 3 integron has been described, and class 4 is limited to *Vibrio cholerae*. Integrons are found almost exclusively in gram-negative bacteria, with one known exception. At least 60 gene cassettes have been described for class 1 integrons. The majority of genes encode antibiotic disinfectant resistance, including resistance to aminoglycosides, penicillins, cephalosporins, trimethoprim, tetracycline, erythromycin, and chloramphenicol (Fletcher and Macrina, 1991). Only a few epidemiological studies using integron-specific PCRs have been described, but in general the gene cassettes present were not characterized due to the wide range of possibilities and combinations. Epidemiological studies of *Enterobacteriaceae* in France and The Netherlands (Sallen *et al.*, 1995; Jones *et al* 1997) showed that over half of the isolates tested carried an integron.

Transposons are well known for their capacity to carry multiple antimicrobial resistance genes, but only a few molecular studies of the epidemiology of transposons have been published. The presence of Tn*7* was often examined in studies using probes to detect trimethoprim resistance. The study by the group in Greece showed that Tn*7* was present in 19 of 57 *E. coli* isolates from persons with urinary tract infections. Tn*7* was most often

associated with DHFR type I (Tsakris *et al.*, 1993). A study of trimethoprim resistance in India also investigated Tn*7* (Tait and Ayres, 1994).

All plasmids carrying DFHR type Ia also hybridized with the Tn*7*-specific probe, whereas plasmids harbouring DHFR type Ib or V hybridized with a probe specific for Tn*21*-like transpsons. A Taiwanese study observed that 7.8% of 374 tri imethoprim resistant isolates also carried Tn*7*, but no link with the DFHR types studied was made (Chang *et al.*, 1992). It is not known whether these isolates harbuor integrons. The best-studied multidrug resistance elements are those which are often harbored by MRSA (Skurray *et al.*, 1988). Australian MRSA isolates may harbor up to three different plasmids: small (1.6-kb) cryptic plasmids, 4.5-kb chloramphenicol resistance plasmids, and 20- to 42-kb plasmids which variously encode resistance to antiseptics and disinfectants, trimethoprim, penicillin, gentamicin, tobramycin, and kanamycin. The last three resistances are encoded on Tn*4001*. The same transposon, although with some slight differences, was also detected in isolates in North America.

Although multidrug resistance is a major problem, the detection of genetic elements which carry multiple resistance genes for either diagnostic or epidemiological purposes is rather limited (Fluit *et al.*, 2001; Branger *et al.*, 2005).

2.11.9.1 *Transposons*

Transposons are also relevant for the dissemination of antibiotic resistance genes, either by integration in transferable plasmids or by direct conjugation and further integration in bacterial chromosome (Bindereif and Neilands, 1983; Martinez *et al.*, 1994). Shiga toxin 1 (VTI) gene has been found in transposable elements in *E. coli* (Hu and Lee, 1988).

Shiga toxin operon has been found in a composite transposon in *Shigella dysenteriae* 1 (McDonough and Butterton, 1999). The toxin of *E. coli* O157:H7, which closely resembles that of *Shigella* dysenteria 1 is called Shiga-like toxin and has been shown to be a transposable element (Gyles, 1992).

2.11.9.2 *Phages*

Bacteriophage-associated transduction of antibiotic resistance determinants has been described (Blahova *et al.*, 1998, 1999). Phages have stringent requirements for the incorporation of novel DNA fragments because of size requirement for phage DNAs. However Shiga toxin is an example of a virulence determinant encoded by phages from *Escherichia coli* O157:H7. *E. coli* O157:H7 phages have not been found to encode for Shiga toxin and antibiotic resistance occurring together (Blahova *et al.*, 1999).

2.11.9.3 *Integrons and Gene Cassettes*

A novel system has been identified in multiple resistant bacteria (MAR) or multiple drug resistance (MDR) bacteria, as the primary system for acquisition and dissemination of antibiotic resistance genes (Hall, 1997, Hall and Stoks, 1993), in Gram-negative bacterial and has also be described in Gram-positive bacteria. Integrons are mobile DNA elements formed by gene cassettes located downstream of a recombinase-encoding conserved sequence that includes a strong promoter. Four classes of integrons have been identified (Mazel *et al.*, 1998).

The majority of integrons identified among clinical isolates belong to a class 1 type (Jones *et al.*, 1997, Sallen *et al.*, 1995). In class 1 integrons, the 5/ conserved region encodes a site-specific recombinase (integrase) and a strong promoter or promoters that

ensure expression of the integrated cassettes. The 3/ conserved segments carries qacΔE, which specifies resistance to antiseptics and disinfectants and an open reading frame. Multiple class 1 integrons have recently been reported in *E. coli* O157:H7 by Zhao *et al.* (2001). The integron was 1kb in size and habuored an aadA gene encoding resistance to streptomycin-spectinomycin. The *E. coli* O157:H7 strain CVM990 (Meng *et al.*, 1998) originated from the USA and was characterized as having *stx1, stx2, eae* and *hlyA* genes and had been isolated from cattle in 1991 (Zhao *et al.*, 2001). The alarm bell is on: Horizontal transfer of the aadA gene was demonstrated to *Hafnia alvei*. The integron-borne aadA gene was silent in *E. coli* whereas it was fully expressed when it was transferred to *H. alvei*, suggesting that horizontal transfer may enhance expression of a resistance gene.

Rao *et al.* (2006) has provided an anticipated response. The group reported class 1 integrons in resistant *Escherichia coli* and *klebsiella* species: A positive test result for class 1 integrons was found for 181 (57%) of 320 bacterial isolates screened including 103 (49%) of 209 *E. coli* isolates and 78 (70%) of 111 *klebsiella* spp. isolates. A positive test result in *E. coli* isolates was significantly associated with non-susceptibility to gentamicin, tobramycin, ciprofloxacin (a fluoroquinolone), chloramphenicol and trimethoprim – sulfamethoxazole. However, multivariate analysis showed that the only drug variable associated with a positive test result for class 1 integrons in *E. coli* were non-susceptibility to gentamicin and trimethoprim –sulfamethoxazole, while an inverse association was present between appositive integron test result and non-susceptibility of cefepime (a 4^{th} - generation cephalosporin).

The prevalence of class 1 integrons in *E. coli* was 49% in selected non - outbreak isolates from hospitalized patients from 2002 to 2004 (Rao *et al.*, 2006) 52% in consecutive urinary tract isolates in 2001 in Sweden (Grape *et al.*, 2005), and 15% from blood in hospitals in Norway 1998 and 1999 (Heir *et al.*, 2004). Other workers have identified class 1 integrons around the world such as Norby, (2005) in Europe. The generally accepted view is that integrons are widely spread in *E.col* and other members of the *Enterobacteriaceae* and the integrons are associated with resistance to antimicrobial agents (Norby, 2005). This has provided an explanation to what has been a well documented but poorly understood attribute of Gram-Negative bacteria: acquiring of drug resistance within and between species (Guiney, 1984) Rao *et al.* (2006) demonstrated that the inverse association between integron presence and non-susceptibility to cafapine in *E. coli* indicates that resistance determinants for the drug may not be frequently carried by integrons. No inverse association was demonstrated for fluoroquinolones. However, what is required is the characterization of a gene carried by an integron for resistance to any of the quinolones or fluoroquinolones. Zhao *et al.* (2005) reported a 1 kb integron in *E. coli* O157:H7 and other STEC *E. coli* haboring a cassette encoding resistance to streptomycin-spectinomycin.

A biologic mechanism for association of resistance to fluoroquinolones and aminoglycosides i.e. the inactivation of ciprofloxacin by the aac(6')-1b-cr aminoglycoside acetylating gene has been recently reported by Robiesek *et al.* (2006), who published that a common animoglycoside acetyltransferase has been adapted and modifies fluoroquinolones.

2.11.10 *Strategies for Addressing Antimicrobial Drug Resistance*

Generally the strategies stress the need for the development of new drugs in the developed countries (CDC, 2001; WHO 2001), and yet the rate of drug development is in decline (Powers, 2004) while antimicrobial drug resistance has become a public health problem (IDSA, 2004, Nordberg *et al.*, 2005).

2.11.10.1 *Antimicrobial Resistance Surveillance and Minimum Inhibitory Concentrations (MIC)*

Regulatory bodies must gather information on antimicrobial resistance patterns to guide usage of the agents. This goes along with data on MIC from these studies to establish limits for prescription (Metlay *et al.*, 2006).

2.11.10.2 *Regulatory Bodies*

Regulatory bodies have roles that can impact positively on the control of resistance in microorganisms. Measures should be based on scientific evidence concerning human health and the effectiveness of interventions. Unfortunately quantitative data on the clinical implications of resistance are lacking for many common infections (Metlay, *et al.* 2006). The clinical implications include symptoms resolution and/or survival (FDA, 1998).

2.11.10.2.1 *Regulatory Responsibility to Resistance*

Regulators are primarily concerned with licensing new drugs or the usage of drugs by verifying their safety, efficacy and quality. Regulators such as NAFDAC (in Nigeria) also have roles that relate to the long term safety of established agents by responding to post-launch data (Metlay *et al.*, 2006).

2.11.10.2.2 *Scheduling*

Most developed countries categorize antimicrobial drugs within a "prescription-only" schedule, thereby preventing over-the-counter sales, giving physicians and other healthcare professionals' responsibility for their distribution (Metlay *et al.*, 2006). This may work in Nigeria if fines are stipulated and enforced against sales and distribution.

2.11.10.2.3 *Prescribing Directives and Information*

Regulators may issue directives to prescribers regarding antimicrobial drug use. This can be advantageous to prevent cross resistance, for example aminoglycosides such as amikacin, gentamicin and tobramycin should be used with restraint together with quinolones in the light of the recent report that a common aminoglycoside acetyltransferase can be adapted to a fluoroquinolone-modifying enzyme. This has to be carefully evaluated to avoid disadvantageous effects on prescribing behaviour (FDA, 2003).

Updating labelling is a substantial undertaking. It is the responsibility of food and drug administrators of individual countries to give labelling requirements for antimicrobial drugs (FDA, 2003). A few countries such as Australia have a registration system for human antimicrobials that incorporate resistant risks assessment. This is the way forward, but as of at now, generic manufacturers have no responsibility to provide resistance data for their products. So, healthcare systems may have to provide resources to collect these data (Metlay *et al.*, 2006).

2.11.10.2.4 *Indication Review*

Indication review is the process by which regulatory authorities reassess the licensed indications of a drug in light of new data. Safety is often the primary consideration but in principle resistance could be used. Any decision to change a drug's license should be based on robust clinical evidence of a public health hazard. In vitro surveillance data may be insufficient in isolation, because uncertainty exist about the threshold resistance prevalence at which indications should be withdrawn (Metlay, *et al.*, 2006).

2.11.10.2.5 *Incentives for Antimicrobial Drug Development and Control*

Collaborations must be encouraged between regulatory agencies, healthcare systems academia, industry and the developing world to identify pathogens of primary public health importance. The development of narrow-spectrum antimicrobials or adjunctive agents that target resistance mechanisms will not be viable without effective low cost diagnostic methods available at the point of prescription including the developing world. Thus, incentives must also be considered for the development and clinical adoption of diagnostic technologies and their availability and usage in developing countries. This includes training, collaborations, and provision of support (Metlay *et al*., 2006).

2.11.11 *Perspectives*

Containing resistance: What we have been privileged to observe over the past 61years since Alexander Fleming's counsel about antibiotics usage is the Darwinian concept of "survival of the fittest" being played out in microbial populations. We have been able to actually watch evolution in progress!! So much has been done about possible ways in which resistance may be overcome. The scrutiny of bacteria genomes has not borne fruit in the much anticipated yield of novel antimicrobial agents. We may have been looking

in the wrong direction. Nature's way is usually simple as it was in the case of the discovery of penicillin (Hamilton-Miller, 2004).

Reducing Antibiotics use: Conventional wisdom has it that this might be achieved by reducing the number of infections by effective prevention and control of infection, immunisation, public health measures at both individual and societal levels, curbing of high risk behaviour and eradication of illiteracy and poverty.

However, possible areas to achieve some measure of success in this area include, refining of dosage regimens, effective scheduling of drugs including the "prescriptions only" model, targeting and improving compliance, restricting prophylactic usage, continuing education of the professionals and the public and reduction of usage outside medical and veterinary practice (Hamilton-Miller, 2004). Less conventional approaches involve application of better understanding of nature in areas like chronobiology and diet.

Alternatives to Antibiotics: A seperate approach to reducing antibiotic use is to bear in mind possible alternatives to antibiotics. Some of these ideas are as follows:

Immunotherapy: Immunotherapy for staphylococcal infections has been widely used in Russia.This has been by applying antistaphylococcal hyperimmune plasma and serum. (Kelly, 2000) This essentially was one of the first responses to the first bacterium to be resistent to penicillin – *S. aureus*. The approach can be tried against several other bacterial species (Hamilton-Miller, 2004)

Bacteriophage therapy: Bacteriophages are viruses that attack bacteria (Hanlon, 2007). Antibiotic resistance has driven the exploration for alternative therapies to revisit the

potential of bacteriophage therapy. Phages cannot be infect mammalian cells and are obligate parasites. A bacteriophage encounters its bacterial host randomly and attaches via special receptors present at the tips of its tail fibres. The phage genome is injected into the bacteria where it is transcribed by the bacterial RNA polymerase into proteins, which redirect the bacterium to manufacture new virus components that are then assembled into complete virions (Hanlon, 2007). Ernest Hankin observed the therapeutic potential of the Ganges river in India, Felix d' Herelle and Frederick Twort noted that it was due to a bacterial virus and d'Herelle named the viruses bacteriophages (bacteria eaters). Bacteriophage therapy promised much but was over shadowed by the antibiotics era in infectious disease therapy. As the quiver containing antibiotics is effectively becoming empty, perhaps, the time has come to revisit phage therapy taking advantage of the subatantial clinical experience of scientists within the former Soviet Union and improved knowledge of bacteriphage biology (Sulakvelidze, 2001; Ahmed 2002 ; Hanlon, 2007)

Bacteriotherapy: Bacteriotherapy is best recognized as the use of probiotics. Probiotics have been reported to be effective in a remarkably wide variety of clinical conditions, including these involving the gastrointerstinal tract (Hamilton-Miller,2004) P. Huovinen has emphasizes that the failure to develop new antibitotics has made bacteriotherapy inevitable (Huovinen, 2001)

Bacterial apoptosis and virulence mechanisms: The discovery that bacteria contain the apparatus to bring about its death perhaps points to the development of novel targets and therapeutic options (Engelberg-Kulka *et al*., 2001). Attacking virulence mechanisms

rather than the whole bacterial structure offers a wide range of possibilities. A great attraction of this type of approach is that, it seems less likely to apply selection pressure that antibiotics have brought to bear on microbial populations. (Hamilton-Miller, 2004) Targets that have been investigated include receptor sites, P-fimbriae and Shiga toxin (Nishikawa *et al.* , 2002).

Complementary and alternative medicine (CAM): These are very widely used by the general public, a fact now recognized by the World Health Organization, WHO in 2002 launched its first global strategy on CAM. The world market is estimated to be worth at least US$60 billion annually (Kessler *et al.*, 2001) Three substances that could be seen to worth exploiting more fully in infectious disease therapy are honey, aloe vera, garlic and tea tree oil (Cox *et al.*, 2000; Cooper *et al.*, 2002)

Can resistance be reversed? : The strategies mentioned above have been devised in order to reduce antibiotic use therapy, leading to decrease rate of emergence of resistance. Reduced selection pressure should have this effect, but to go further and actually bring about a decline in the incidence of resistance requires more radical measures, as many resistant bacteria seem to be as fit as their sensitive counterparts (Sander *et al.*, 2002). Certain lines of research offer some optimism that this could be achieved. Blocking of a known, existing resistance mechanism will convert resistance to sensitivity. This has been reported in the case of certain B-lactamases by claulanate, sulbactam and tazobactam. (Hamilton- Miller, 2004). A similar type of approach is reported of the tea component epicatechin gallate on methicillin-resistant *Staphylococus aureus,* whereby production of PBP2 is suppressed and sensitivity to methicillin regained. (Yam *et al.*,

1998) Inhibition of the efflux pumps that confer resistance to tetracycline, fluoroquinolones and other antibiotics, with consequent antibiotic reversals of resistance has been reported (Renau and lemoine, 2001). The tea component epigallocatechin gallate inhibits the transferof conjugative R plasmids both in vitro (Potera, 2001) and in animal feeding experiments. (Van den Bogaard *et al.*, 2002) A suprisingly neglected finding is that the antimalarial quinacrine preferentially killed bacteria containing R-plamids in *E. coli* (Yoshikawa and Sevag, 1967). So why are we not exploring these CAM sources at individual and community levels?

Post – antibiotics era: Some authorities have seriously suggested the end of the antibiotic era (Cohen, 1991), although resistance rates continue to creep upwards this seems unnecessarily pessimistic, in the short term at least (Hamilton-Miller, 2004). Bacteria are indeed versatile: can survive for thousands of years as spores, grow at extremes of temperature 0^0C (for psychrophiles), 100^0C for (thermophiles), extremes of pH, pH 0 for *Acetobacter,* pH 9 for *Vibrio cholerae,* utilize substances toxic to man and animals (H_2S, NH_3, CO and methanol), and uses penicillins as a sole source of Carbon (N-*Burkhokderia cepacia* and *Pseudomonas fluorescens)* (Hamilton-Miller *et al*., 2004). However, pitted against the human brain, when eventually, as it seems inevitable, antibiotics fail to offer therapeutic efficacy against certain agents of infectious diseases, alternative measures will have been found. Dr Alexander Fleming, the 1945 Nobel laureate in medicine for the discovery of penicillin once put it as being destiny (Fleming, 1945). Some call it human ingenuity (Hamilton-Miller, 2004)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sampling

3.1.1 *Sampling Methods*

Using the multistage (cluster) sampling method (Snedecor and Cochran, 1976), Kaduna and Sokoto states were selected by a simple random sampling from seven states in the North-west geo-political zone namely: Jigawa, Kaduna, Kano, Katsina, Kebbi, Sokoto and Zamfara. Four of six dairy farms in Kaduna State and two of four dairy farms in Sokoto State were selected in the second stage of the multistage sampling method by a second simple random sampling.

3.1.2 *Sampling Sites and Sample Collection*

Sampling was started in October 2000 and was completed in May 2006.The selected dairy farms in Kaduna State were: Farms A, B, C and D (Appendix 1). In Sokoto State the selected dairy farms were E and F. Non-dairy cattle were randomly selected for sampling including cattle at slaughter at the Zango abattoir in Kaduna State; Sokoto abattoir in Sokoto State; Beef farms (G, H and I) (Appendix 1) in Kaduna State and ten nomadic Fulani cattle herds (five in each state) around Zaria and Sokoto Cities in Kaduna and Sokoto States respectively.

An introductory letter was delivered to each farm manager, explaining the aims of the study and requesting for permission to collect faecal and milk samples from cattle and water samples meant for consumption by the cattle (Appendix 2).

Samples of 20g to 50g of faeces were collected from the rectum of each animal by rectal palpation and retrieval with a clean gloved hand, with adequate restraint of the animal (Chapman *et al.* 1994; Zhao *et al.*, 1995). Each sample was labelled and a questionnaire (Appendix 3) was administered for each animal soliciting information on the animal's ear-tag number and/or nickname, age, breed, sex, location of the farm (farm name), management type, presence of diarrhea, nature of diarrhea, duration of diarrhea, number of animals in the herd, number currently with diarrhea, history of diarrhea in the herd, source of water available to the animals, other conditions observed on the animal and history of antimicrobial use on each animal. Samples were not collected from animals that recently received (one month interval) or were undergoing antimicrobial therapy. Each sample was held in a plastic bag which was knotted and kept in a cool box containing ice blocks until all the cattle at a selected site were sampled. At the sampling sites in the field, approximately 10g of each faecal sample was enriched in 90ml of mTSB supplemented with novobiocin (Oxoid, Basingstoke England) (Appendix 4) (Zhao *et al.*, 1995). A total of 1800 animals were sampled.

Milk samples were collected from each quarter of the udder just before each animal was milked after the udder was cleansed with a mild soap, rinsed with warm water and dried by the farm's personnel (hand or machine milking). Up to 5ml of milk was collected from the milkier and inoculated into sterile wide-mouth type bottles already containing 45ml of the enrichment medium-modified Trytone Soya Broth (mTSB) (Oxoid Ltd, Hamphire England), supplemented with Novobiocin (Oxoid, Hampshire England) (Chapman *et al.*,

1993a). A total of 400 milk samples comprising of 350 unpasteurized and 50 bulk-milk samples were collected.

A total of 360 water samples from water intended for animal use was sampled by collecting 200ml of untreated, borehole and treated (tap) water. Abattoir effluents from Zaria, Kaduna State and Sokoto municipal area were sampled and 300 samples were collected during the surveys. The samples were collected aseptically using sterile beakers and syringes and held in sterile bottles in a cool box containing ice blocks. Up to 10 mls of the abattoir effluents were enriched in bottles already containing MTSB supplemented with novobiocin (Oxoid, Basingstoke England) (Coghlan *et al*., 1975).

Imported cheese and minced meat from Australia were obtained from an importer in Zaria. The samples were collected, wrapped in sterile paper and transported to the laboratory.

3.2 Isolation of SMAC Negative *E. coli*

3.2.1 *Faecal Samples*

Escherichia coli O157:H7 was isolated as described by Zhao *et al.* (1995) with minor modifications. Briefly, approximately 10g of faeces was enriched in 100mls of modified Tryptone Soya Broth (mTSB) supplemented with novobiocin (Oxoid Ltd, Hampshire England) (Appendix 3). This enrichment phase had been carried out in the field soon after sample collection. On arrival at the laboratory the enriched samples were incubated at 37^{O}C for 24hours, and cultured on sorbitol Macconkey agar (Appendix 5)

supplemented with cefixime (CR-SMAC) (Oxoid Basingstoke, England), and incubated at 37^{O}C for 24 hours (Zhao *et al.*, 1995).

A hundred and ninety-three of the initial faecal samples were inoculated without the enrichment phase onto Eosin Methylene Blue (EMB) (Appendix 6) agar and incubated at 37^{O}C for 24 hours. Colonies showing a greenish metallic sheen on EMB were selected and screened biochemically. Isolates that were biochemically identified as *E. coli* were then plated on CR-SMAC agar. Colourless colonies on CR-SMAC were selected and stored on nutrient agar (NA) slants at 4^{o}C until required for serological confirmation.

3.2.2 *Milk Samples*

The enriched milk samples were plated on CR-SMAC agar (Appendix 4) and incubated at 37^{O}C for 24 hours. Colourless colonies on CR-SMAC agar were streaked on NA slants and stored at 4^{O}C (Al-Gallas *et al.*, 2002).

3.2.3 *Water Intended for Animal Use*

In the laboratory, each water sample was tested for detection of faecal contamination using the membrane filtration technique (Coghlan *et al.*, 1975) with modifications: 10 to100ml water samples (depending on source) were filtered through membrane filter with pore sizes of 0.45um. The organisms on membrane filters were then enriched in mTSB. Each enriched membrane filter was cultured on CR-SMAC agar supplemented with novobiocin (Oxoid Basingstoke England), for the isolation of *E. coli* O157:H7 by

selection of typical colourless colonies. The colonies selected were stored on NA slants at 4^{o}C.

3.2.4 *Abattoir Effluents*

After enrichment of the abattoir effluents samples (in the field), each sample was incubated at 37^{o}C for 24 hours and plated onto CR-SMAC agar and incubated at 37^{O}C for 24 hours in the laboratory. Typical colourless colonies on CR-SMAC agar were streaked on NA slants and stored at 4^{O}C (Coghlan *et al*., 1975).

3.2.5 *Cheese and Mince Meat*

A 30g portion of each food sample was homogenized in a stomacher (Lab Blender 400, Seward Medical,London,England) with 270ml of mTSB (Appendix 3) and incubated at 37^{o}C for 24 hours in the laboratory. The enriched samples were plated on CR-SMAC agar. Typical colourless colonies on CR-SMAC agar were streaked on NA slants and stored at 4^{O}C until required for biochemical tests (Al-Gallas *et al.*, 2002).

3.3 Biochemical Screening

The stored selected colonies were screened biochemically using procedures described in detail by Coghlan *et al.* (1975). Each isolate was tested for citrate utilization, urease production, H_2S production in triple sugar iron (TSI) agar and sulphide indole motility (SIM) medium, motility and indole production using SIM medium, fermentation of glucose, sucrose and /or lactose in TSI medium, methyl red (MR) and Voges – Proskauer (VP) reactions using MRVP medium, ability to produce an acid or a non-acid reaction

from fermentation of lactose, arabinose maltose, sorbitol and manitol, and gas from glucose (Appendix 7-12).

3.4 Presumptive Identification of *E. coli* O157:H7

The Remel Wellcolex *E. coli* O157:H7 kit (Remel Europe Ltd, Dartford Kent, U.K) was usedt to identify CR-SMAC negative isolates that were biochemically *E. coli*. Wellcolex *E. coli* O157:H7 is a rapid latex agglutination test for the presumptive identification of *E. coli* O157:H7 isolates on laboratory media. The test kit contains two test reagents. The somatic (O157) antigen test reagent consists of red latex particles coated with antibodies specific for *E. coli* (O) antigen. When a drop of the reagent is mixed on a card with a suspension of *E. coli* O157 organisms, rapid agglutination occurs through the interaction of specific IgG and O157 lipopolysaccharide antigen. Similarly the H7 test reagent consists of blue latex coated with antibodies specific for the flagellum, (H7) antigen. (Appendix15).

The instructions of the manufactures concerning choice of media and laboratory practice were followed as specified. The instructions are issued out in a leaflet that accompanies the kit. The performance of the test and control latex reagents was confirmed using fresh overnight cultures of a control strain EHEC EDL 933.

Haemolytic activity of the serologically confirmed isolates was tested by culturing on brain heart infusion (BHI) agar containing 7% human blood and incubating at 37^{o}C for

24 hours. (Appendix13). The haemolitic pattern was observed and interpreted basing on clear haemolyisis on the agar at the point of inoculation (Coghlan *et al* 1975)

3.5 Shiga toxin Production by *E. coli* O157

The production of Shiga toxins 1 and 2 (Stx1 and 2) by isolates, that were presumptively identified to be *E. coli* O157 was carried out using the Vero Toxin *Escherichia coli*-Reverse Passive Latex Agglulination (VTEC-RPLA) test kit (Oxoid Ltd, Hampshire England) (Appendix 14).

Polymyxin B (Oxoid Ltd, Basingstoke, England) at a concentration of 5,000 units per ml was used to facilitate the release of the shiga toxins (verocytotoxins) (Karmali *et al*., 1989) and the solid culture method was followed. Briefly serologically confirmed isolates of *E. coli* O157:H7 were inoculated onto brain heart infusion (BHI) agar (Oxoid Ltd, Hampsrire England) slopes (10ml volumes) and incubated at 37^{o}C for 20 hours. From a pure culture on BHI agar two colonies of the organism to be tested were transferred using a Pasteur loop and suspended in 1ml of 0.85% saline containing 5000 units of polymixin B. The two colonies were colourless, similar in morphology and were picked successively without flaming the loop between removing separate colonies. This ensures viability and vigour of the test organism (Bauer *et al*., 1966). Extraction was carried out for 30 minutes at 37^{O}C in a vibrating water bath. Each culture extract was refrigerated at 4^{O}C for 30 minutes and then centrifuged at 4000 rpm for 20 minutes. The extract was used to assay for Stx1 and Stx2 following the manufacture's instructions. (Appendix13).

3.6 Polymerase Chain Reaction (PCR) Assay for Characterization of Bovine Isolates of E. coli O157:H7

Three sets of PCRs were carried out, multiplex, duplex, and single primer PCR. The M-PCR targeted *rfbE*$_{O157}$ and *fliC*$_{H7}$, the genes that define *E. coli* O157:H7 serotype and the *stx*$_{2d}$ gene which encodes a component of the major virulence factor-the Stx2 in *E.coli* O157:H7 (Wang *et al*., 2002). The D-PCR targeted *fliC*$_{H7}$ and *stx*$_{2d}$ genes (Wang *et al.*, 2002), and the PCR targeted the *stx*$_1$ gene (Al Gallas *et al*., 2002)

3.6.1 *Bacterial Strains*

Serologically confirmed Shiga toxin-producing isolates of *E.coli* O157:H7 from faeces were used for the M-PCR assay. Milkborne *E.coli* O157:H7, O157:H- and faecal O157:H7 were used for the D-PCR assay. A single Stx1 producing *E.coli* O157:H7 and other *E.coli* O157:H- isolates of faecal origin were used for the PCR assay.

3.6.2 *Isolation of DNA*

The method of Silhavy *et al*. (1984) was used. Total DNA was extracted from bacterial isolates grown overnight in brain heart infusion (BHl) broth. Briefly, 1 ml of BHl broth culture was pipetted and centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded and the pelleted sediment was re-suspended in 500 ml Tris- ethylene diamine tetraacetic acid (Tris-EDTA)-(TE) buffer (pH8.0) and frozen at –20^{o}C. Then 50µl of lysozyme solution was added and thawed in a vibrating water bath. The thawed harvested cells were put on ice for 45 min and then 100 µl of STEP (1-µg/µl proteinase K, in STE

buffer (pH 7.5) was added and mixed thoroughly. The mixture was left at 50^{o}C in a water bath overnight

This was followed by the addition of 600µl phenol/chloroform/isoamyl alcohol (25:24:1) with gentle mixing for 5 minutes to emulsify. The mixture was centrifuged at 12000 rpm for 10 minutes. The DNA was observed in the top aqueous layer which was transferred to a clean tube without the interface. Then 60µl of 0.1 vol. of 3 M sodium acetate was added and mixed gently to precipitate the DNA and 1200µl (2 vol.) of absolute ethanol was added and the tubes inverted to mix. A centrifuging step at 12,000 rpm for 10 min. precipitated DNA as pellets. The pellets were re-dissolved in 200µl TE buffer (pH 7.5). The solution was washed in 70% alcohol, centrifuged and the supernatant discarded and a block heater was used to dry up the DNA. The pellets were finally dissolved in 30µl of nuclease free water.

3.6.3 *Primers Used in the Study*

The primers used were RfbE, FliC, Stx2d and Stx1 (Operon, Cologne Germany). The primer sequences used (Table 3.1), are specific for the *rfbE*$_{O157}$, *fliC*$_{H7}$, *stx*$_{2d}$ and *stx*$_{1}$ respectively (Al- Gallas *et al.*, 2002; Wang *et al.*, 2002)

3.6.4 *Multiplex-PCR Conditions*

The primer mix for the M-PCR and D-PCR were based on Wang *et al* (2002) and the manufacturers instructions. The primer mix for the PCR was based on Al-Gallas *et al.*(2002).

The M-PCR was done in a total volume of 25 µl containing 2.5µl of 10 x buffer (100 mM Tis-HCl at pH 8.3, 500 mM KCl), 0.5 µl of Taq DNA polymerase (Eppendorf, Hamburg Germany), 1.5µl $MgCl_2$, 0.5 µl deoxynucleoside triphosphate, 0.5 µl *fliC-a*, 0.5 µl *fliC-b*, 1.88 µl *rfbE-a*, 1.88 µl *rfbE –b*, 1.88 µl stx_{2d}-*a*, 1.88 µl stx_{2d}-*b*, and 5 µl of crude DNA extract. The volume of the mix was adjusted to 25 µl with 6.48 µl of nuclease free water. This M-PCR was the characterisation of faecal isolates of *E.coli* O157:H7. DNA amplification was carried out in a Techne Cambridge Thermocycler (Cyclogene, USA), using an initial denaturation step at 95^{o}C for 8 minutes, followed by 30 cycles of amplification with denaturation step at 95^{o}C for 30 seconds, annealing at 58^{o}C for 30 seconds and extension at 72^{o}C for 30 seconds, ending with a final extension at 72^{o}C for 7 minutes (Wang *et al.*, 2002). Aliquots of 10 µl of amplified products were analysed by electrophoresis in 2% agarose gels (Seakem, Rocklaid Maine, USA). Electrophoresis was run at 80V for 30 minutes. A loading dye and a 50 bp DNA ladder (SIGMA, St. Louis Missouri, USA) were used. The gels were stained with ethidium bromide and the PCR products were visualized under ultra –violet (UV) transillumination (Fotodyne Inc, Heartland WS, USA) and photographed using MP-4 land camera and digital devices. Positive samples were identified based on the presence of bands of appropriate sizes of PCR amplicons in base pairs (bp) (Table 3.1).

The second protocol used was based on Wang *et al.* (2002) and the manufactures instructions as previously described. However, only FliC and Stx2d were used as primers. Positive samples were identified based on the presence of bands of appropriate sizes of

PCR amplicons in base pairs (bp) (Table 3.1). This D-PCR was for the characterization of faecal *E.coli* O157:H7 and a milkborne *E. coli* O157:H7 and H- isolates.

In the third protocol, the Stx1 primer mix was based on Al-Gallas *et al.* (2002) and the instructions of the manufacturer. The PCR mixture consisted 2.5 µl of 10x Buffer, 1.50 µl of $MgCl_2$, 0.50 µl deoxynucleoside triphosphates, 0.40µl stx1 –a, 0.40 Stx 1-b, 0.50, Taq DNA polymerase (Ampli Taq Gold, Applied Biosystems) and 3 µl of template DNA. The volume of this mix was adjusted to 25 µl by addition of 16.2 µl of nuclease free sterile water. DNA amplification was carried out in a Gene Ampl. PCR system 9700 (Applied Biosystems California, USA) using an initial denaturation step at 94^{o}C for 5 minutes, followed by 30 cycles of amplification with denaturation step at 94^{o}C for 30s, annealing at 53^{o}C for 30s and extension at 72^{o}C for 30s ending with a final extension at 72^{o}C for 10 min and cooled to 4^{o}C. This PCR assay was for the detection of the *stx*1 gene from a faecal isolate of *E.coli* O157:H7 and *E.coli* O157:H-

Table 3.1: Primers used for the Detection of *rfbE, fliC, stx$_{2d}$* and *stx1* Genes.

Primer	Primer Sequence (5' to 3')	Target Gene	Size of PCR Amplicon (bp)
Stx2d-a	GGTAAAATTGAGTTCCTCAAGTAT		
Stx2d-b	CAGCAAATCCTGAACCTGACG	*stx*$_{2d}$	175
RfbE-a	CTACAGGTGAAGGTGGAATGG		
RfbE-b	ATTCCTCTCTTTCCTCTGGGG	*rfb*E$_{O157}$	327
FliC-a	TACCATCGCAAAAGCAACTCC		
FliC-b	GTCGGCAACGTTAGTGATACC	*fliC*$_{H7}$	247
Stx1-a	GAAGAGTCCGTGGGATTACG		
Stx1-b	AGCGATGCAGCTATTAATAA	[a]*Stx*$_1$	130

Wang *et al.* (2002)

[a]Pollard *et al.* (1990)

3.7 Antimicrobial Resistance Testing

In vitro susceptibility tests were performed by the standardized single disk diffusion method (Bauer *et al.*, 1966) in Mueller-Hinton agar (MHA) (Oxoid, Basingstoke, England) with 16 antimicrobial disks using a recommended *E.coli* O157:H7 reference strain as the control organism. The antimicrobial agents tested and their disk concentrations in micrograms were the following: those commonly used in veterinary medicine including ampicillin (AMP10), streptomycin (C10), gentamicin (CN10), kanamycin (K30), neomycin (N10), tetracycline (TE30), sulphamethoxazole (RL25), trimethoprim/sulphamethoxazole1:19 (co-trimoxazole) (SXT25), compound sulphonamides (S3 300); and antimicrobials employed mainly in humans including cephazolin (KZ30), ceftazidime (CAZ30), cefuroxime (CXM30), ciprofloxacin (CIP5), nalidixic acid (NA30) norfloxacin (NOR10) and chloramphenicol (C30) (Oxoid, Basingstoke, England) (Ramon – Yusuf *et al.*, 1990; Mora *et al.*, 2005).

From a pure culture of the organism on SMAC agar, 5 colonies of the organism to be tested were transferred with a wire loop to a test tube containing 4ml of Tryptone Soya Broth (TSB). All five colonies were colourless, similar in morphology and were picked successively i.e without flaming the loop between removing separate colonies.

The tubes were incubated at 37^{o}C for 2 hours. The suspension was standardized by diluting with 0.85% saline to a density visually equivalent to the McFarland Standard 0.5 (a turbidity standard prepared by adding 0.5 ml of a 1% barium chloride solution to 99.5 of 1% H_2SO_4). This equates to approximately 10^8 *Enterobacteriaceae* organisms per ml. Mueller-Hinton Agar (MHA) plates were prepared and dried in an incubator. Cotton

swabs were immersed into the culture and the entire surface of MHA was spread evenly with the suspension. The innoculum was allowed to dry with the plates covered for 10 minutes. Using aseptic techniques each disc was placed and pressed gently to ensure full contact with the agar surface. Immediately the inverted plates were incubated at 37^{o}C for 14 hours.

The diameter of the zone of inhibition of growth including the diameter of the disk was measured to the nearest millimeter (mm) by use of a ruler. The zone produced by each isolate was compared with that produced by the control strain (EDL 933).

A strain was considered sensitive if the diameter of its zone of inhibition of growth by the antimicrobial agents was greater than, equal to, or not more than 4mm less than that on the control culture. The average diameter of the inhibition zone produced by the control strain was 16 mm. A strain was moderately sensitive if its zone diameter was 11-12 mm and resistant if it showed no zone of inhibition of growth or if the diameter of its zone was not more than 10 mm (Coughlan *et al.*, 1975)

3.8 Statistical Analysis

The data generated from the microbiological and serological screening was sent into Mcrosoft Excel 2003 (Microsoft Corp., Redmond, WA, USA) and descriptive analysis was performed. The files were imported into the statistical package for social sciences (SPSS) for Windows 11.0 (Standard Version SPSS Inc., Chicago, IL, USA). Chi-square and Fisher's exact tests were used to test for significance ($p<0.05$) (Snedecor and Cochran, 1976).

CHAPTER FOUR

4.0 RESULTS

4.1 Prevalence of SMAC Negative (Presumptive *E. coli* O157) from Cattle, Food and the Environment

The prevalence of SMAC negative *E. coli* from cattle faecal samples was 12.6% (227/1800), 8.6% (30/350) from fresh (unpasteurized) milk and 22% (11/50) for bulk milk. The prevalence was 3.3% (10/300), 12% (55/460), 10% (2/20) and 0% (0/20), for abattoir effluents, water intended for animal use, minced meat and cheese respectively. The overall prevalence of SMAC negative *E.coli* from cattle was 10% (268/2240); 12.6% (227/1800) from faecal shedding, 8.6% (30/350) from fresh milk and 22% (3/50) from bulk milk. The over all prevalence from enviromental samples was 9.8% (65/660); 3.3% (10/300) for abattoir effluents and 12% (55/360) for water intended for animal use (Table 1).

The prevalence of SMAC negative *E.coli* from cattle faeces was not dependent on the state where sampling was carried out (Pearson χ^2, p=6.515) (Table 4.2); the location of sampling sites within the states (Pearson χ^2, p=70.313) (Table 4.2); the year that the samples were collected Pearson χ^2, p=38.777) (Table 4.3); the season (Pearson χ^2, p=3.950) (Table 4.4); the age of the animals (Pearson χ^2, p=1.189) (Table 4.5); the sex (Pearson χ^2, p=15.688) (Table 4.6); breed (Pearson χ^2, p=27.413) (Table 4.7); management (Pearson χ^2, p=34.743) (Table 4.8); the quality of water given to the animals (Pearson χ^2, p=0.520) (Table 4.9); and diarrhea status of the animals (Pearson χ^2, p=1.841) (Table 4.10).

Table 4:1: Prevalence of CR-SMAC Negative Presumptive *E. coli* O157 from Cattle, Food and the Enviroment.

Sample	No. Examined	No. Negative	Prevalence
CATTLE			
Bovine Faeces	1800	227	12.6%
Fresh Cow Milk	350	30	8.6%
Bulk Cow Milk	50	11	22%
SUB-TOTAL	**2,200**	**268**	**10%**
ENVIRONMETAL			
Abattoir Effluents	300	10	3.3%
Water [a]	360	55	12%
SUB-TOTAL	**660**	**65**	**9.8%**
Minced Meat	20	2	10%
Cheese	20	0	0%

[a] Water intended for animal consumption

Table 4.2: Distribution of CR-SMAC Negative Isolates based on State and Location (sampling sites)

State	Sampling Sites (Location)	CR-SMAC		
		Positive	Negative	Total
Kaduna	Farm A	333	66	399
	Farm B	253	54	307
	Farm C	78	13	91
	Farm D	38	20	58
	Zaria Abattoir; Farms G, H and I; Nomadic White Fulani Cattle Herds, Zaria	624	53	677
Sub-total		**1328**	**206**	**1530**
Sokoto	Farm E	139	18	157
	Farm F	45	1	46
	Sokoto Abattoir and Nomadic White Fulani Cattle Herds, Sokoto	63	2	65
Sub-total		**247**	**21**	**268**
GrandTotal		**1573**	**227**	**1800**

CR-SMAC: Cefixime-sorbitol MacConkey agar.

Table 4.3: Distribution of CR-SMAC Negative Isolates Based on Year of Sample Collection.

	CR-SMAC		
Year	Positive	Negative	Total
2002	196	5 (2.2%)	201
2003	880	135 (59.5%)	1015
2004	75	5 (2.2%)	80
2005	239	32 (14.1%)	271
2006	183	50 (22.0%)	233
Total	1573	227 (100%)	1800

CR-SMAC negative is presumptively *E. coli* O157:H7

Table 4.4: Distribution of CR-SMAC Negative Isolates Based on Season of the Year.

	CR-SMAC		
Season	Positive	Negative	Total
Dry (November-February)	692	84 (37%)	776
Rainy (May-October)	881	143 (63%)	1024
Total	1573	227 (100%)	1800

CR-SMAC negative is presumptively *E. coli* O157:H7

Table 4.5: Distribution of CR-SMAC Negative Isolates Based on Age Group.

	CR-SMAC		
Age Group	Positive	Negative	Total
Young Cattle[a]	557	72 (32%)	629
Adult Cattle[b]	1016	155 (68%)	1171
Total	1573	227 (100%)	1800

CR-SMAC negative is presumptively *E. coli* O157:H7

a ≤ 1 year old

b > 2 years old

Table 4.6: Distribution of CR-SMAC Negative Isolates Based on Sex.

	CR-SMAC		
Sex	Positive	Negative	Total
Males	616	58 (26%)	674
Females	957	169 (74%)	1126
Total	1573	227 (100%)	1800

CR-SMAC negative is presumptively *E. coli* O157:H7

Table 4.7: Distribution of CR-SMAC Negative Isolates Based on Breed.

	CR-SMAC		
Breed	Positive	Negative	Total
Exotic[a]	789	156(69%)	945
Local[b]	784	71 (31%)	855
Total	1573	227 (100%)	1800

CR-SMAC negative is presumptively *E. coli* O157:H7

[a] Imported foreign breeds of cattle

[b] Indigenous Nigerian breeds of cattle

Table 4.8: Distribution of CR-SMAC Negative Isolates Based on Management System.

	CR-SMAC		
Management System	Positive	Negative	Total
Intensive (Dairy)	868	172 (76%)	1040
Extensive	705	55 (24%)	760
Total	1573	227 (100%)	1800

CR-SMAC negative is presumptively *E. coli* O157:H7

Table 4.9: Distribution of CR-SMAC Negative Isolates Based on Water Quality.

	CR-SMAC		
Water Quality	Positive	Negative	Total
Good[a]	345	45(20%)	390
Poor[b]	1228	182 (80%)	1410
Total	1573	227 (100%)	1800

CR-SMAC negative is presumptively *E. coli* O157:H7

[a] Treated (tap water), rain and borehole water

[b] Stream, lake, pond, dam and shallow well water

Table 4.10: Distribution of CR-SMAC Negative Isolates Based on Health Status.

	CR-SMAC		
Health Status	Positive	Negative	Total
Diarrhea	218	24 (11%)	242
No Diarrhea	1355	203 (89%)	1558
Total	1573	227 (100%)	1800

CR-SMAC negative is presumptively *E. coli* O157:H7

4.2 Prevalence of Serologically (Remel Wellcolex Test) Presumptively Identified *E. coli* O157 in Cattle

The prevalence of remel Wellcolex indentified *E. coli* O157 was 1.6% (28/1800) for cattle faeces, 0% (0/350) for fresh cow milk and 6% (3/50) for bulk cow milk. There was no isolation from abattoir effluents, water intended for animal use, minced meat and cheese (Table 4.11)

The prevalence of remel Wellcolex *E. coli* O157 from cattle faeces was not significantly different between the states (Pearson χ^2, p=4.202). The prevalence was also not dependent on location (Pearson χ^2, p=50.113), year of study (Pearson χ^2, p=14.692), age (Pearson χ^2, p=3.653), sex (Pearson χ^2, p=1.880), breed(Pearson χ^2, p=7.753), water quality available to the animals (Pearson χ^2, p=3.307) and diarrhea status of the animals (Fishers exact test, p= 0.532) but was dependent on the management type (Fisher's exact test, p= 0.000).

4.3 Serotypes and Prevalence of Remel Wellcolex Test Presumptively Identified *E. coli* O157:H7 from Cattle

Two serotypes were identified: *E. coli* O157:H7 and *E. coli* O157: H-. The prevalence of Remel Wellcolex *E. coli* O157:H7 from cattle faeces was 0.9% (17/1800) and 2% (1/50) for bulk cow milk while the prevalence of *E. coli* O157: H- from faeces was 0.7% (11/1800) and 4% (4/50) from bulk milk (Table 4.11).

The prevalence of *E.coli* O157: H7 from cattle faeces was not dependent on the state where sampling was carried out (Pearson χ^2, p=5.639) (Table 4.12); season of the year (Pearson χ^2, p=7.330) (Table 4.13); age (Fishers Exact test, p=0.235) (Table4.14); sex (Pearson χ^2, P=0.473) (Table4.15); water quality (Pearson χ^2, p=6.520) (Table 4.16) and

diarrhea status (Fisher's exact test, p=0.038) (Table 4.17) but was assotiated significantly with breed (Fisher's exact test, p=0.038) (Table4.18) and management type (Fisher's exact test, p=0.001) (Table 4.19).

4.4 Prevalence of Shiga Toxin Production by Remel Wellcolex Test Identified *E. coli* O157 from Cattle

The prevalence of Shiga toxin producting *E. coli* O157 in cattle was 97% (30/31).

The toxins were Shiga toxin 1 (Stx1) which was detected in 3% (1/30) and Shiga toxin 2 (Stx2) which was detected in 97% (29/30) of Shiga toxin producing isolates (Table 4.20).

Table 4.11: Prevalence and Serotypes of Remel Wellcolex Test Identified *E. coli* O157:H7 from Cattle.

Sample Type	Number Examined	Wellcolex O157 Positive[a][d]	Serotype[d]	
			O157:H-	O157:H7
Faeces	1800	28(1.6)	11(0.7)	17(0.9)
Fresh Milk	350	0(0)	0(0)	0(0)
Bulk Milk	50	3(6)	2(4)	1(2)
Abattoir Effluent	300	0(0)	0(0)	0(0)
Water[b]	260	0(0)	0(0)	0(0)
Minced Meat[c]	20	0(0)	0(0)	0(0)
Cheese[c]	20	0(0)	0(0)	0(0)

[a] Remel Wellcolex *E. coli* O157:H7 kit (Remel, Europe Ltd, Kent, UK).

[b] Water intended for cattle consumption

[c] Imported from Australia

[d] Figures in parentesis are prevalences in percentages

Table 4.12: Distribution of presumptively identified *E. coli* O157:H7 based on States

	REMEL Wellcolex *E. coli* O157:H7 Test		
State	Positive	Negative	Total
Kaduna	11	1521	1532
Sokoto	6	262	268
Total	17	1783	1800

The minimum expected count = 2.53

Pearson Chi-Square, P = 5.639

Table 4.13: Distribution of presumptively identified *E. coli* O157:H7 based on season of isolation

	REMEL Wellcolex *E. coli* O157:H7 Test		
Season	Positive	Negative	Total
Dry	5	771	776
Wet	12	1012	1024
Total	17	1783	1800

The minimum expected count = 7.33

Pearson Chi-Square, P = 1.313

Table 4.14: Distribution of presumptively identified *E. coli* O157:H7 based on age

	REMEL Wellcolex *E. coli* O157:H7 Test		
Age	Positive	Negative	Total
Young Cattle	4	625	629
Adult Cattle	13	1158	1171
Total	17	1783	1800

The minimum expected count = 5.98

Fisher's exact test, P = 0.235

Table 4.15: Distribution of presumptively identified *E. coli* O157:H7 based on Sex

	REMEL Wellcolex *E. coli* O157:H7 Test		
Sex	Positive	Negative	Total
Female	12	1114	1126
Male	5	669	674
Total	17	1783	1800

The minimum expected count = 6.37

Pearson's Chi Square, P = 0.473

Table 4.16: Distribution of presumptively identified *E. coli* O157:H7 based on the quality of water fed to the cattle.

	REMEL Wellcolex *E. coli* O157:H7 Test		
Water quality	Positive	Negative	Total
Poor	9	1401	1410
Good	8	382	390
Total	17	1783	1800

The minimum expected count = 3.68

Pearson's Chi Square, P = 6.520

Table 4.17: Distribution of presumptively identified *E. coli* O157:H7 based on Health (diarrhea status) of the cattle.

	REMEL Wellcolex *E. coli* O157:H7 Test		
Diarrhea Status	Positive	Negative	Total
Absent	15	1543	1558
Present	2	240	242
Total	17	1783	1800

The minimum expected count = 2.29

Fisher's exact test, P = 0.594

Table 4.18: Distribution of presumptively identified *E. coli* O157:H7 based on breed of cattle.

	REMEL Wellcolex *E. coli* O157:H7 Test		
Breed	Positive	Negative	Total
Exotic	13	932	945
Local	4	851	855
Total	17	1783	1800

The minimum expected count =8.07

Fisher's exact test, P = 0.038

Table 4.19: Distribution of presumptively identified *E. coli* O157:H7 based on management type.

	REMEL Wellcolex ***E. coli* O157:H7 Test**		
Management	Positive	Negative	Total
Dairy	16	1024	1040
Extensive	1	759	760
Total	17	1783	1800

The minimum expected count = 7.18

Fisher's exact test, P = 0.001

Table 4.20: Production of Shiga-toxins by Remel Wellcolex identified *E. coli* O157 from Cattle.

Origin.	No. Examined	No. Positive (VTEC-RPLA Assay)	Shiga toxin 1 (Stx1)	Shiga toxin 2 (Stx2)
Faecal Samples	28	27	1	26
Bulk Milk	3	3	0	3
Total	31	30	1	29

1.5 Detection of *stx$_{2d}$*, *fliC* and *rfbE* Genes by Multiplex and Duplex-PCR

A multiplex-PCR assay was run to detect *stx$_{2d}$*, *fliC* and *rfbE* genes (Figure 4.1; Table 4.21) from faecal isolates of *E.coli* O157:H7 and a duplex-PCR to detect *stx$_{2d}$* and *fliC* (Figure 4.2) from faecal and bulk-milk isolates of *E.coli* O157:H7 and H- (Table 4.22).

Ten of the one thousand eight hundred faecal isolates were positive for *E. coli* O157:H7 by multiplex-PCR (Table 4.21). This gave the prevalence of faecal shedding of *E. coli* O157:H7 in cattle by multiplex-PCR to be 0.6% (10/1800); and the prevalence in bulk milk was 2% (1/50) (Table 4.22), from the single confirmed *E.coli* O157:H7 by D-PCR (Figure 4.2)

The prevalence by multiplex-PCR of *E. coli* O157:H7 in cattle (faeccal origin) was significantly associated with season (Fisher's Exact Test, p=0.030) (Table 4.23); age (Fisher's Exact Test, p=0.046) (Table 4.24) and management (Fisher's Exact Test, p=0.034) (Table 4.25). However, the detection of *E. coli* O157: H7 defining genes were independent of diarrhea (Fisher's Exact Test, p=0.602) (Table 4.26); water quality (Fisher's Exact Test, p=0.627) (Table 4.27); breed (Fisher's Exact Test, p=0.440) (Table 4.28) and sex (Fisher's Exact Test, p=0.448) (Table 4.29).

Only ten (59%) of the 17 isolates of faecal origin serologically confirmed to be *E. coli* O157:H7 were actually genotypically *E. coli* O157:H7 (Figure 4.1). Six (41%) were *E. coli* O157:H- (Figure 4.2) and one was a non-O157 (Figure 4.1). The one *E. coli* O157:H7 serologically identified from bulk milk was confirmed by D-PCR (Figure 4.2) making the prevalence of *E. coli* O157:H7 by D-PCR in bulk milk 2% (1/50). All the 11

PCR confirmed isolates of *E. coli* O157:H7 except one were from apparently healthy cattle.

4.6 Detection of Shiga Toxin Genes

The detection of Shiga toxin genes was in 60% (12/20) of the serologically confirmed isolates of *E. coli* O157:H7 (Figures 4.1, 4.2). Nine of the twelve were isolates from faecal samples and three were isolates from bulk milk. The detection of Shiga toxin genes from serologically confirmed isolates of faecal origin was 53% (9/17) and 100% (3/3) since all three bulk milk samples tested harboured the Shigatoxin gene (Tables 4.30, 4.31).

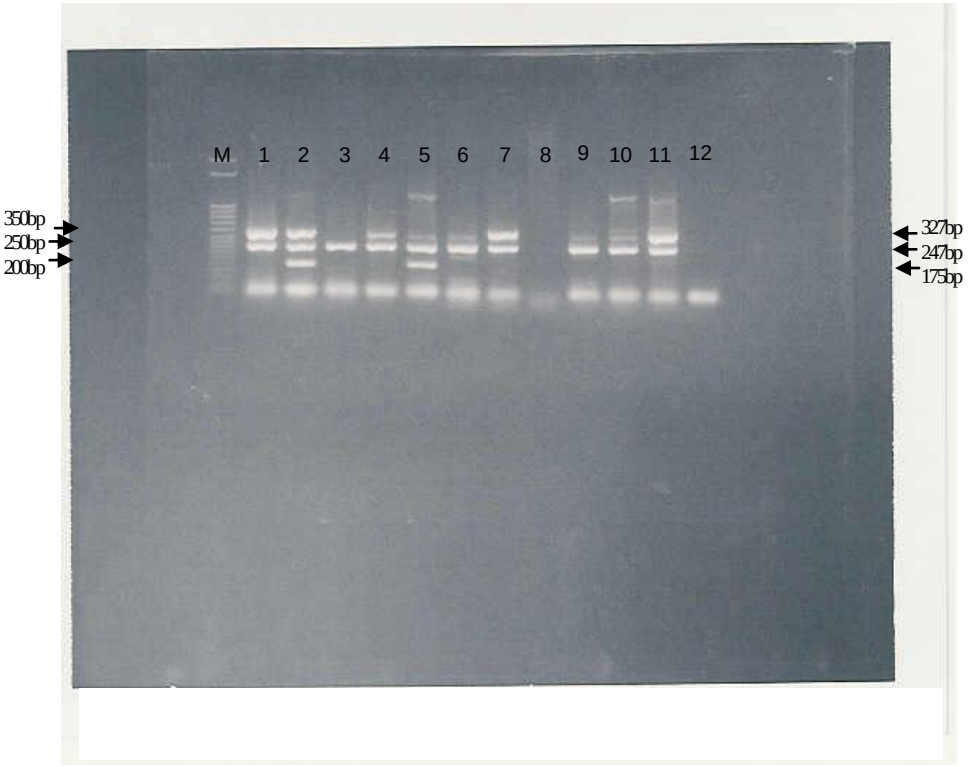


Figure 4.1: M-PCR Profiles of *E.coli* O157:H7 from Cattle (Faecal)

Lane M 50bp DNA Ladder (Sigma St Loiuse, Missouri USA)
Lane 1 *E.coli* O157:H7
Lane 2 *E.coli* O157:H7 Habouring the stx_{2d} gene
Lane 3 *E.coli* O157:H7
Lane 4 *E.coli* O157:H7
Lane 5 *E.coli* O157:H7 Habouring the stx_{2d} gene
Lane 6 *E.coli* O157:H7
Lane 7 *E.coli* O157:H7
Lane 8 *E.coli* O157:H7
Lane 9 *E.coli* O157:H7
Lane 10 *E.coli* O157:H7
Lane 11 *E.coli* O157:H7
Lane 12 Negative control *Salmonellena typhimurium*

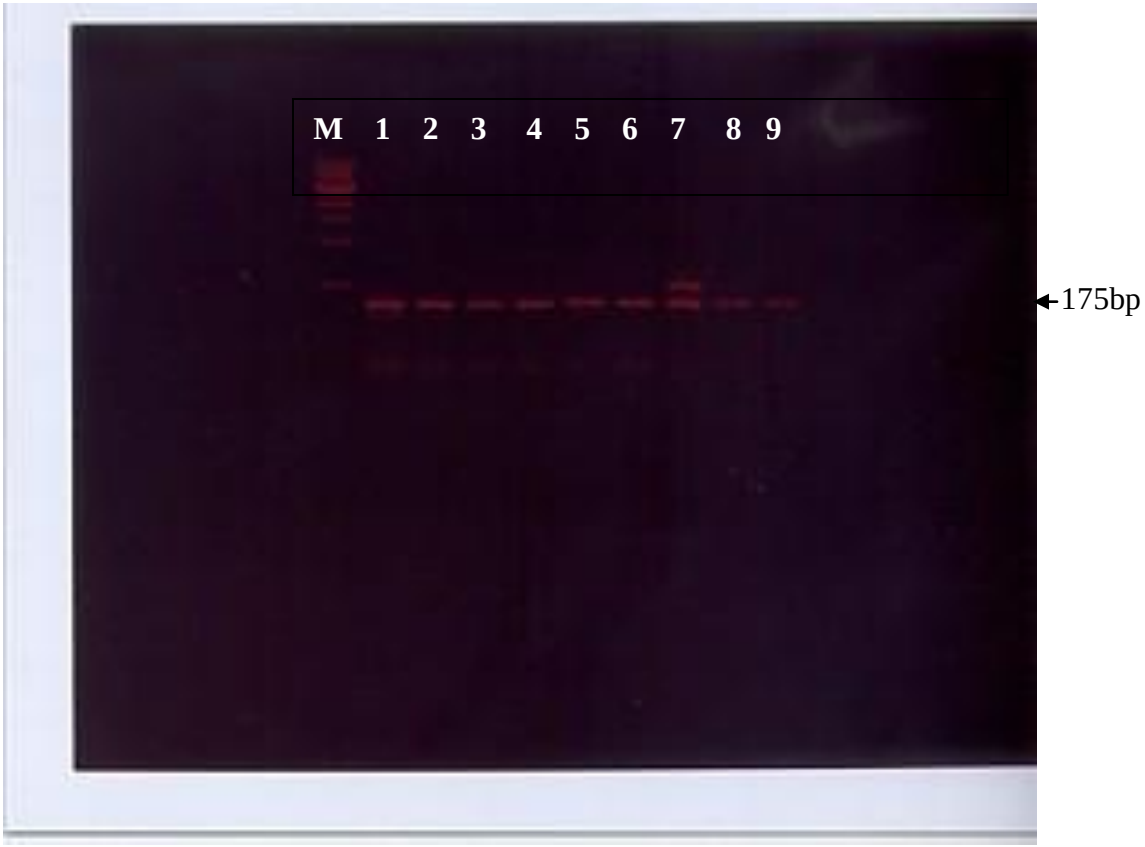


Figure 4.2: D-PCR Amplification Profiles from Faecal and Bulk Milk Isolates.

Lane M: 1kb ladder (Bio Labs, New England, USA)
Lanes 1-6 *stx*$_{2d}$ (175bp)
Lane 7 *stx*$_{2d}$ (175bp), fliC(247bp)
Lanes 8-9 *stx*$_{2d}$ (175bp)

Table 4.21: Characterization of *E. coli* O157:H7 Isolates of Faecal Origin by Multiplex-PCR.

S/No	Sample Number	Strain[a](Well Number)	Age	Sex[b]	Breed[c]/Origin	Genes detected
1	6	KD102 (10)	1m	M	F-B, (Dairy farm A)	[o]*rfbE, fliC*
2	484	KD202(1)	3m	M	F-B, (Dairy farm A)	[f]*rfbE, fliC*
3	494	KD303 (11)	10m	M	B (NWF Biye KDS)	[o]*rfbE, fliC*
4	1494	KD405 (9)	6 yr	F	F (Dairy farm B)	[o]*rfbE, fliC*
5	1499	KD505 (6)	6 yr	F	F (Dairy farm B)	[o]*rfbE, fliC*
6	1514	KD605 (2)	5 yr	F	F(Dairy farm B)	[o]*rfbE, fliC* stx_{2d}
7	1664	KD706 (3)	1 yr	F	SG (Dairy farm D)	[o]*rfbE, fliC*
8	1667	KD806 (4)	1 yr	F	F-B (Dairy farm D)	[o]*rfbE, fliC*
9	1680	KD906 (7)	1 yr	F	B (Dairy farm D)	[o]*rfbE, fliC* stx_1
10	1655	'(8)	3yr	F	M-SG (Dairy farm D)	None
11	1750	KD1006(5)	1 yr	F	F-B (Dairy farm D)	[o]*rfbE, fliC* stx_{2d}

[a]Well numbers; [c]F, Friesian; F-B, Friesian –Bunaji Cross; B, Bunaji, SG, Sokoto Gudali; NWF, Nomadic White Fulani, M-SG [b]M, Male; F, Female [f]diarrhea [o]apparently healthy

Table 4.22: Characterization of E. coli O157:H7 of Faecal and Bulk Milk Isolates by Multiplex –PCR

S/No	Sample Number	Strain[a]	Age	Sex[c]	Breed/Origin[d]	Genes detected
1	985	1 03 (2)	3yr	M	F, Farm F, Dairy	*Stx*$_{2d}$
2	986	2 03 (3)[b]	4yr	F	F, Farm F, Dairy	*Stx*$_{2d}$
3	1003	3 03 (4)[b]	3 yr	F	F, Farm F, Dairy	*Stx*$_{2d}$
4	1032	4 03 (5)[b]	4yr	F	F, Farm F, Dairy	*Stx*$_{2d}$
5	1034	5 03 (6)[b]	5yr	F	F, Farm FD, Dairy	*Stx*$_{2d}$
6	984	6 06 (7)[b]	6yr	F	F, Farm D, Dairy	*Stx*$_{2d}$
7	8	KD1106(8)[b]	-	-	B-milk, Farm D, Dairy	*stx*$_{2d}$, *fliC*$_{H7}$
8	3	7 06 (9)[b]	-	-	B-milk, Farm D, Dairy	*Stx*$_{2d}$
9	10	8 06 (10)[b]	-	-	B-milk, Farm D, Dairy	*Stx*$_{2d}$

[a] The figures in parenthesis are well numbers during electrophoresis of PCR products.

[b] See figure 4.3

[c] M, Male; F, Female

[d] B, Bunaji, B-milk, Bulk-milk, F, Friesian, S, Simmental.

Table 4.23: Distribution of characterized *E. coli* O157:H7 based on season of Isolation.

	PCR		Total
Season	Positive	Negative	
Dry	1	775	776
Wet	9	1015	1024
Total	10	1790	1800

The minimum expected count = 4.31

Fisher's exact test, P=0.0.030

Table 4.24: Distribution of characterized *E. coli* O157:H7 based on age

	PCR		Total
Age	Positive	Negative	
Young Cattle	3	626	629
Adult Cattle	7	1164	1171
Total	10	1790	1800

The minimum expected count = 3.49

Fisher's exact test, P=0.046

Table 4.25: Distribution of characterized *E. coli* O157:H7 based on management type.

	PCR		Total
Management	Positive	Negative	
Dairy	9	1031	1040
Extensive	1	759	760
Total	10	1790	1800

The minimum expected count = 4.22

Fisher's exact test, P=0.034

Table 4.26: Distribution of characterized *E. coli* O157:H7 based on diarrhea status of the cattle.

	PCR		Total
Diarrhea Status	Positive	Negative	
Absent	9	1549	1658
Present	1	241	242
Total	10	1790	1800

The minimum expected count = 1.34

Fisher's exact test, P=0.602

Table 4.27: Distribution of characterized *E. coli* O157:H7 based on the quality of water fed to the cattle.

	PCR		Total
Water quality	Positive	Negative	
Poor	8	1402	1410
Good	2	388	390
Total	10	1790	1800

The minimum expected count = 2.17

Fisher's exact test, P=0.627

Table 4.28: Distribution of characterized *E. coli* O157:H7 based on breed of cattle

	PCR		Total
Breed	Positive	Negative	
Exotic	6	939	945
Local	4	851	855
Total	10	1790	1800

The minimum expected count = 4.75

Fisher's exact test, P=0.440

Table 4.29: Distribution of characterized *E. coli* O157:H7 based on Sex

	PCR		Total
Sex	Positive	Negative	
Female	7	1119	1126
Male	3	671	674
Total	10	1790	1800

The minimum expected count = 8.74

Fisher's exact test, P = 0.448

4.7 Detection of *stx$_1$* by PCR

A PCR assay was run to detect *stx$_1$* gene. Only one isolate was *stx$_1$* positive (Figure 4.3).

4.8 Antimicrobial Resistance Among *E. coli* O157 from Cattle

Twenty-one of 28(75%) *E. coli* O157 isolates were resistant to at least one of the antimicrobials. Resistance to Sulphamethoxazole was the most common antimicrobiol resistance with 18/28 (64%), followed by compound sulphonamides 14/28(50%), tetracycline 10/28(36%), ampicillin 6/28(21%), streptomycin 6/28(21%), nalidixic acid 5/28(18%), sulphamethoxazole / trimethoprim 5/28(16%), cephtaxidime 3/28(11%), neomycin 2/28(7%) and cefuroxime, cephazolin, kanamycin, gentamicin and chloramphenicol 1/28(4%). Sulphonomide resistance was the highest of all classes of antimicrobials tested (64%), followed by tetracycline (36%), aminoglycosides (21%) β-lactams (21%) and quinolones (18%). There was no resistance to the flouroquinolones (Table 4.30).



Figure 4.3: Profiles of PCR Amplicons for Amplification of the *stx*$_1$

Lane M: 50bp DNA ladder (Roche)
Lane 3: *stx*$_1$ (130bp)
All other lanes negative for the *stx* gene

Table 4.30: Antimicrobial resistance of presumptively identified *E. coli* O157 by class of antimicrobial.

Class/Antimicrobials	Resistance Strain / Total Investigated	Percentage Resistant Isolates
B-Lactams		
Ampicillin	6/28	21
Cephalosporins		
Ceftazidime	3/28	11
Cefuroxime	1/28	4
Cephazolin	1/28	4
Chloramphenicol	1/28	4
Tetracycline	10/28	36
Aminoglycosides		
Kanamycin	1/28	4
Gentamicin	1/28	4
Streptomycin	6/28	21
Neomycin	2/28	7
Sulfonamides and/or trimethoprim		
Sulfamethoxazole	18/28	64
Co-trimoxazole	5/28	10
Compound Sulphonamides	14/28	50
Quinolones		
Nalidixic acid	5/28	18
Flouroquinolones		
Ciprofloxacin	0/28	0
Norfloxacin	0/28	0

4.9 Antimicrobial Resitance of PCR Confirmed Isolates of *E. coli* O157:H7 from Faeces

Sulphamethoxazole was the most common antimicrobial resistance observed at 8/10(80%), followed by compound sulphonamides 6/10(60%), co-trimoxazole tetracycline, ampicillin and ceftazidime 3/10(30%), cefuroxime, cephazolin, gentamcin, nalidixic acid and neomycin 1/10(10%). There was no resistance to kanamyicin, chloramphenicol, ciprofloxacin and norfloxacin. Sulphonamide resistance was the highest of all classes (80%), followed by tetracyclines, β-lactams, (30%), aminoglycosides and quinolones (20%). There was no resistance to chloramphenicol and the fluoroquinolones (Table 4.31).

Table 4.31: Antimicrobial resistance of mulitiplex - PCR Confirmed isolates of *E. coli* O157:H7 by class of Antimicrobial.

Class/or Antimicrobial	ResistantStrains/ Total Investigated	Percentage Resistant Strain
β-Lactams		
Ampicillin	3/10	30
Cehpalosporins		
Ceftazidime	3/10	30
Cefuroxime	1/10	10
Cehpazolin	1/10	10
Chloramphenicol	0/10	0
Tetracycline	3/10	30
Aminoglycosides		
Streptomycin	2/10	20
Neomycin	1/10	10
Gentamicin	1/10	10
Kanamycin	0/10	0
Sulfonamides and/or trimetoprim		
Sulfamethoxazole	8/10	80
Compound Sulphonamides	6/10	60
Sulphamethoxazole/trimetoprim	3/10	30
Quinolones including (flouroquinolones)		
Nalidixic acid	1/10	20
Norfloxacin	0/10	0
Ciprofloxacin	0/10	0

4.10 Predominant Antimicrobial Resistance Patterns of 28 Isolates of *E. coli* O157 from Cattle

Table 4.32 shows all the 21 antimicrobial resistance patterns found among the 28 isolates tetsted. Multiple drug resistance (MDR) was detected in 18/28(64%) of *E. coli* O157 isolates tested. The MDR patterns most frequently observed were AMP-CN-S-RL-S3-TE-SXT 3/28(11%).Seven isolates (25%) were not sensitive to any antimicrobial agent tested (Table 4.32).

Table 4.32 Predominant Patterns of Antimicrobial Resistance of 28 Isolates of *E. coli* O157 From Dairy Cattle.

Resistance Pattern	No. of Isolates (%)
AMP, NA, S, RL, S3, TE, SXT	3 (10.9)
RL, S3	2 (7.1)
RL	2 (7.1)
RL,S3,TE,SXT	2(7.1)
CAZ, CXM, RL, S3	1 (3.5)
CAZ	1 (3.5)
CAZ, RL, S3	1 (3.5)
AMP, KZ	1 (3.5)
AMP, CN, S, RL, S3, TE, SXT	1 (3.5)
RL, TE	1 (3.5)
NA, RL, S3	1 (3.5)
RL, S3, TE,	1 (3.5)
AMP, C, S, RL, TE	1 (3.5)
S, TE	1 (3.5)
RL, S, TE	1 (3.5)
K, NA, N, S, RL, S3, TE	1 (3.5)
Sensitive to all antimicrobials tested	7(25.0)

Overall, 21 resistance patterns were observed.

CHAPTER FIVE

5.0 Discussion

Escherichia coli O157 grew on Sorbitol-MacConkey (SMAC) agar as colourless colonies reaffirming the suitability of SMAC agar as a selective/differential medium for the primary isolation of this organism from faecal, food and environmental samples.

The use of SMAC agar provided a rapid screening method for investigating *E. coli* O157 in cattle and the presence of the organism in food and the environment. Typical (colourless, smooth, circular and entire edged) colonies were apparent on SMAC agar after incubation at 37^{o}C for 12 hours. This screening method was especially suitable because it is relatively cheap when compared to more rapid, specific and sensitive methods such as Microarray analysis of *E. coli* O157:H7 (Jin *et al.*, 2005), Whole Genome PCR scanning (Ogura *et al.*, 2006), Rapid Pulsed-Field Gel Electrophoresis for detecting *E. coli* O157: H7 in 1 day (Gautom, 1997), Direct enumeration of *E. coli* in water by 16S rRNA *in situ* hybridization (Torterello and Reineke, 2000) and Immunomagnetic separation (IMS) (Chapman and Cudjoe, 2001).

In this study, the sensitivity of SMAC agar was improved by initial enrichment before culturing. The specificity of the enrichment medium and SMAC agar was improved by addition of specific selective supplements novobiocin for modified tryptone soya broth (MTSB) and cefixime for SMAC agar (CR–SMAC agar). This protocol was described by Zhao *et al.* (1995) and variations of it have been used extensively (Al-Gallas *et al.*, 2002; Paiba *et al.*, 2003;Cagney *et al.*, 2004; Normano *et al.*, 2004; Keen *et al.*, 2006).

Presumptively identified *E. coli* O157 based on colonial morphology on SMAC agar, were biochemically screened as *E. coli*, because other coliforms such as some *Citrobactor freundii* strains also may ferment D-sorbitol slowly and so appear colourles on SMAC agar after incubation at 37^{o}C for 18-24 hours (Bettelheim, 1993). Moreover, inability to ferment sorbitol is an easily transferable plasmid-encoded trait within the enterobacteriaceae and other Gram-negative bacteria such as *Brucella* (Lior and Borcyzk, 1987). Isolation methods for *E. coli* of varying specificities are often used depending on the objective of the study. Bartoloni *et al.* (2006) used MacConkey agar without an enrichment phase and with no biochemical or presumptive screening to isolate *E. coli* in children from Peru and Bolivia. He studied multi-drug resistance (MDR) in commensal *E. coli* demonstrating the use of basic media. However, the low prevalence of *E. coli* O157 from fresh milk, water, abattoir effluents, minced meat cheese and faecal samples when compared to the high prevalence of presumptively identified *E. coli* O157 using only CR-SMAC agar emphasizes the importance of biochemical and serological screening of isolates of *E. coli* O157 from these sources. There was a statistically significant difference between the prevalence of presumptively identified CR-SMAC negative *E. coli* O157 isolates from faecal samples 12.6% (227/1800) and the prevalence when a latex agglutination method was used to detect the pathogen from faecal samples at 1.6% (28/1800) (χ^2, p=0.031). This finding is consistent with the use of CR-SMAC as just an initial screening step in the identification of *E. coli* O157:H7 from faecal, food and enviromental samples (Zhao *et al.*, 1995; Al-Gallas *et al.*, 2002; Paiba *et al.*, 2003; Cagney *et al.*, 2004; Normano *et al.*, 2004; Keen *et al.*, 2006).

The identification of *E. coli* O157:H7 and *E. coli* O157:H- has confirmed the emergence of the pathogen in the cattle population in Northwestern Nigeria, since the pioneering work of Tekdek (1994) identified the O?, O8, O15, O75, O26 and O6 serogroups in calves. The emergence of *E. coli* O157: H7 in the cattle population in this part of the country is of public health significance because over 40% of the Nigerian cattle population is from this zone (Bourn *et al.,* 1994). This organism is zoonotic and a major cause of out-breaks of bloody diarrhea when it infects humans, with fatal complications in children and the aged.

The initial foci of reservoirs appear to be dairy cattle, since the organism was mostly isolated from apparently healthy dairy cattle but the pathogen has also spread to nomadic white Fulani cattle. The prevalence of *E.* coli O157:H7 of 0.9% and 0.6% by Remel Wellcolex test and PCR assays from faecal samples respectively is within the range reported by studies from other countries (Chapman, 1997; Martin *et al*., 1986; Orskov *et al.*, 1987; *Wells et al.*, 1991; Garber *et al.*, 1995). The organism was isolated and confirmed serologically from 66.7% (4/6) of dairy farms surveyed but was present in only Kaduna state. There was confirmation of the entry of *E. coli* O157:H7 into the Nigerian food chain with the isolation and confirmation from milk. The prevalence of *E. coli O*157: H7 in dairy cattle from the USA has been reported as ranging from less than 1to 10% on farm based surveys (Armstrong *et al.*, 1996). Higher prevalence has been reported from cattle on farms with outbreaks of the infection in human farm workers and in cattle at agricultural fairs in the USA. The prevalence reportedly ranged from 6.4% in cattle at trade fairs to over 40% in cattle on farms habouring human diarrhea cases of *E.*

coli O157: H7 (Armstrong *et al.*, 1996; Keen *et al.*, 2006). The situation is similar in England and Wales where Paiba *et al.* (2003) reported a 4.2% prevalence of excretion by individual cattle from a survey of cattle farms. These reports show that *E. coli* O157: H7 is entrenched in the cattle population of these countries. The prevalence of 0.9% from this study is on the lower side of the range in farm cattle. However the methods used for isolating *E. coli* O157:H7 from samples greatly influences the prevalence. Paiba *et al.* (2002) used a variation of the Zhao *et al.* (1995) method that was used in the present study and revealed a prevalence rate of 4.2% in England and Wales. This lower prevalence indicates the emerging nature of the pathogen in Northwestern Nigeria. The organism was also found in a bulk-milk sample on one dairy farm but could not be isolated from fresh milk, abattoir effluents, water fed to cattle, minced meat and cheese. This could be due to low count of the organism in these samples. The pathogen needs to be up to 10^2 CFU/g in a sample to be positive by the direct plating procedure (Zhao *et al.*, 1995).

Two serotypes of *E. coli* O157 were identified by our study; *E. coli* O157:H7 and *E. coli* O157: H- using the Remel Wellcolex *E. coli* O157:H7 test. The prevalence of *E. coli* O157:H7 from faecal samples based on the Remel Wellcolex *E. coli* O157:H7 test was not dependent on season, age, breed, sex, diarrhea, management and water quality. The non-specificity of the latex agglutination test may have accounted for this observation. Two multiplex – PCR assays were used to characterize the 17 presumptively identified enterohaemorrhagic Shiga toxin – producing *E. coli* O157: H7 and *E. coli* O157: H- from faecal and bulk milk by the detection of stx_1, stx_{2d}, $fliC_{H7}$ and $rfbE_{O157}$ genes. However,

the prevalence of M-PCR confirmed isolates of *E. coli* O157:H7 was significantly associated to age, season and management type. Hiefers and weaned calves were the major age group that the organism was confirmed from. This may be due to the stress on this age group arising from weaning, changes in diet and movement with the herd in search of pastures. Changes in management of cattle on dairy farms have been proposed to have created the environment that favours the survival of the *E. coli* O157: H7 as well as enhancing its virulence. The changes include early grain feeding and contamination of feed and pastures. Grain feeding alters the acid balance in the rumen making the pathogen acid resistant and so more virulent to man (Garber *et al.,* 1995). Heavy rains lead to extensive contamination of pastures and water and the lush pasture creates conditions in the gastrointestinal tract of young animals that enhance faecal excretion (Effler *et al.,* 2001).

The findings of this study on age, seasonality (wet season) and dairying being associated to faecal excretion of *E. coli* O157:H7 are similar to the findings of several studies on the pathogen (Garber *et al.*, 1995; Zhao *et al.*, 1995; Effler *et al.*, 2001; Paiba *et al.*, 2003). Stress induced by changes in the diet and seasonal variations have been proposed as factors that are responsible for the increased excretion by dairy young cattle (Sharma and Beuchat, 2004).

The independence of faecal excretion from breed is complimentary to the thinking that it is the management practices that enhance excretion.The quality of water that is fed to cattle was also not a significant factor and this could be as a result of massive faecal

contamination of all water sources available to the cattle on these farms. Most of the farms visited did not cover the water that was fed to the animals. Almost all farms indicated that their animals consume water from ponds and streams.

The prevalence of *E. coli* O157 from bulk-milk samples of 6% using the Remel Wellcolex *E. coli* O157 latex agglutination test is alarming because contamination of milk by *E. coli* O157 renders it un-fit for human consumption, since the infective dose of *E. coli* O157 in humans is very low, ranging from 2 – 10 cells (Coia, 1998; Willshaw *et al.*, 1994). Detection by latex agglutination tests used by this study and by Luga *et al.* (2006) in a study of dairy cattle in Kaduna State is a less sensitive and specific method when compared to the molecular based methods used by Padhye and Doyle (1991) in their study of bulk-milk tanks in Tennessee, USA. Therefore, the 6.0% isolation of this present study and Luga *et al.* (2006) should be viewed as being high, even though Padhye and Doyle (1991) reported a prevalence of 10%using PCR based assays.

Serious outbreaks of *E. coli* O157: H7 infections have been associated with the consumption of raw or unpasteurized milk (Armstrong *et al*., 1996; FSAI, 1999). This is because *E. coli* O157: H7 apart from having a low infective dose may be present in milk, even when produced under apparently hygienic conditions as a result of post-milking faecal contamination on the farm. The sale of unpasteurized cow's milk for direct consumption is illegal in many countries (FSAI, 1999) but it has not been outlawed in Nigeria.

The present study did not isolate *E. coli* O157: H7 from water, abattoir effluents, fresh milk, minced meat and cheese. This may be due to low counts of the organisms in these samples. The pathogen needs to be up to 10^2 CFU/g in a sample to be positive by direct plating procedures (Zhao *et al.*,1995) This viewpoint is supported by Armstrong *et al.*(1996) who reported in their review that isolation is greatly influence by the sensitivity of the method used for primary isolation of the organism. Outbreaks of *E. coli* O157: H7 involving water, unpasteurized milk, cheese and ground beef have been described by several authors. Effler *et al.* (2001) reported on the association of outbreaks of *E. coli* O157: H7 and water. Cagney *et al.* (2004) reported on *E. coli* O157: H7 in ground beef. Abdul-Raouf *et al.* (1996) and Coia *et al.* (2001) have reported isolation of *E. coli* O157: H7 from raw cow's milk.

The study has revealved that dairy and indigenous white Fulani cattle are reservoirs of *E. coli* O157:H7 and *E. coli* O157:H- The isolation of *E. coli* O157:H- from apparently healthy cattle faeces and fresh milk samples should be of concern to the public, dairy and fast food industries because these pathogens cause diarrhea and death in man. Contamination of water and food by organisms from bovine reservoirs is reported as the major mode of transmission to younger animals and man. A huge reservoir of this zoonotic pathogen could be building up in the cattle population from infected susceptible stock through the consumption of contaminated water and milk. Shedding has been shown to commence when such reservoirs are subjected to stress such as a change in diet or drought (Effler *et al.* 2001). Effler *et al.* (2001) confirmed *E. coli* O157: H- as being

genotypically similar to *E. coli* O157:H7. This study could not confirm the isolates and report them as *E. coli* O157: H- identified by the Remel Wellcolex *E. coli* O157:H7 test.

Production of Shiga toxins has confirmed the isolates as pathogenic, belonging to the STEC group. This finding has a public health significance because STEC strains have been associated with outbreaks of disease that include HC and HUS in humans. The two categories of *E. coli* Shiga toxins Stx1 and Stx2 were identified. Shiga toxin 1 (Stx1) in 3% and Stx2 was detected in 97% of Shiga toxin producing isolates tested. A similar ratio has been reported by Paiba *et al.* (2003). The production of both Stx1 and Stx2 by an isolate was not observed in this study. It has however being reported by Paiba *et al.* (2003). STEC O157:H7 has been reported as being responsible for over 90% of the cases of HUS in North America. Some researchers have implied that STEC O157:H7 is the sole cause of HUS in children (http://www.about-ecoli.com/page3.html). It has been shown epidemiologically that Stx2 is more critical than Stx1 for the development of HUS. *E. coli* O157:H- strains and other non – O157 STEC have been shown to produce Shiga toxins (Wang *et al.*, 2002). Shiga toxin production is an attribute of *E. coli* that depends on the environment. This has been used to explain the non-pathogenicity of STEC to its cattle reservoirs, the elaboration of toxins in susceptible host such as man and its displacement from the rumen by a medium containing prebiotic sugars (De Vaux *et al.* 2002; Zhao *et al.* (1998). Increasing the ruminal acidity has been reported as making the organism acid – resistant and so more pathogenic to man. Grain feeding has been incriminated as the major cause of increased acid – resistance in *E. coli* O157:H7 (Diez-Gonzalez *et al.*, 1998; Russel *et al.*, 2000).

The findings of this study confirmed that cattle in northwestern Nigeria may act as reservoirs of this zonotic pathogen, and raw (unpasteurized) cow's milk as a source of *E. coli* O157:H7 and *E. coli* O157: H-. Only 59% (10/17) of *E. coli* O157: H7 were confirmed by M-PCR. The single *E. coli O*157:H7 isolate from bulk milk was confirmed by M-PCR. These findings appear to indicate that antigenic typing assays should only be used as screening tests for the identification of *E. coli* O157:H7 and *E. coli* O157:H-; since *E. coli* O157 has several non – H7 serotypes such as O157: H10, H19, H21, H43 and H45 and appear to cross react with other serotypes and organisms(Lior and Borcyzk, 1987; Bettelheim, 1993).

Identification of stx_{2d} and *stx1* was carried out by multiplex – PCR and PCR. Only 30%(3/10) of the multiplex – PCR confirmed faecal isolates harboured Shiga toxin genes. Eleven isolates haboured the stx_{2d} and one harboured the stx_1 genes. Toxin testing of *E. coli* O157:H7 is often not carried out, because virtually all the strains produce Shiga toxins. So the other O157:H7 strains could be habouring the genes encoding for the four other subtypes of Shiga toxin 2. Eleven subtypes of Shiga toxin 2 have been identified with different genes (Bertin *et al.,* 2001; Gobius *et al*., 2003). They share a lot of homology in their sequences and often occur together (Zhang *et al*., 2005). This may explain the occurrence of stx_{2d} in the present study since stx_{2d} has not been reported in cattle. The Stx2 initially recognized as the other Shiga toxin, other than Stx1is now one of eleven subtypes of stx_2. To conclude that an isolate is not Shigatoxigenic, all the 11 variants of stx_2 would have to be tested for and testing for stx_1.

The present study reports that sulphonamide resistance was the highest of all antimicrobial agents tested (68% for sulphamethoxazone and 45% for compound sulphonamides); followed by tetracycline (32%); ampicillin (19%), the only β-lactam tested; and aminoglycosides, (19% for streptomycin); and was the highest of all classes of drugs tested. This agrees with the conclusions that *E. coli* strains are more resistant to the most commonly used antimicrobial agents in animal production and veterinary medicine (Mora *et al.*,2005).These data raises important questions about the impact of antimicrobial use in animal production and health and the entry of resistant pathogens into the food chain in N.W. Nigeria. The high resistance to sulphonamides, tetracycline, ampicillin and streptomycin is not likely to have been secondary to administration of these antimicrobials to patients with diarrhea or other ailments since such high levels are only observed when the antimicrobials are directly used in animals (Mora *et al.*, 2005). Moreover, streptomycin is nephrotoxic and has largely been replaced by newer aminoglycosides. As bovine are the principal reservoir of STEC, and since these antimicrobials are used extensively in cattle in Northwestern Nigeria (Ramon-Yusuf *et al.* 1990), it may be assumed that selection pressure that is brought to bear on microbes from the use/misuse of antimicrobial agents in cattle production and health may have created a pool of resistant phenotypes in cattle in Northwestern Nigeria.

However, among the multiplex-PCR confirmed isolates of *E. coli* O157:H7 resistance to ceftazidime a cephalosporin was as high as ampicillin. There was resistance to cefuroxime and cephazolin the two other cephalosporins used. Resistance to

cephalosporins is medediated by extended-spectrum B-lactamases (ESBL), which are now observed world wide in all species of *Enterobacteriaceae* (Gniadkowski, 2001). In almost all instances, ESBL are derieved by point mutations from β-lactamases. A new family of ESBLs the cefotaximase extended spectrum beta-lactamases (CTX-M-ESBLs) have become particulary widespread and are mainly found in *salmonella* and *E. coli*. ESBLs are usually described as acquired β-lactamases that are encoded mainly by genes located on plasmids. Some ESBL-encoding genes are located within transposons or integrons which facilitate transfer between organisms even without any external selection pressure. Organisms with ESBLs pose a therapeutic challenge, since they are frequently resistant to other kinds of antimicrobials including aminoglycosides and sulphonamides (Branger *et al.* 2005). The association between ESBLs and integrons has provided a novel explanation to the rapid spread of antimicrobial resistance in bacteria (Bradford 2001).

Resistance to Nalidixic acid has being observed in isolates of E. coli O157 from cattle (Schroeder *et al.,* 2002). Since nalidixic acid is not used for the treatment of infections in cattle, resistance to it by bovine isolates *E.coli* O157:H7 has been attributed to integrons which usually disseminate the resistance together with several other classes of drugs, leading to multiple drug resistance. Resistance to nalidixic acid is important from a public health view point. Nalidixic acid is the type quinolone, a class of frontline antibiotics in infectious disease therapy. It is not recommended for therapeutic purposes in veterinary practice. The quinolones target two bacterial enzymes, DNA gyrase and topoisomerase IV. Mutations remove the site of quinolone activity from *gyrA* and *gyrB* genes in *E. coli*,

making the organism resistant to quinolones. These mutations are inherent and can be induced by bacterial proteins like Microcin B17 (Drlica and Zhao, 1997). It had been reported that single mutation in *gyrA* gene of *E. coli* may be sufficient to cause high-level resistance to nalidixic acid (Ozeki *et al.*, 1997).

Multiple drug resistance (MDR) was observed in the present study in *E. coli* O157:H7, including resistance to ampicillin (a β-lactam), gentamicin (an aminoglycoside), sulphamethoxazole (sulphonamide) and nalidixic acid (aquinolone). This pattern of resistance is suggestive of the involvement of novel resistance mechanisms that have been reported as being responsible for the wide spread resistance that is being observed bacteria in the 21st century. These mechanisms include integrons and extended spectrum β-lactamase (ESBL) producing *Escherichia coli* O157:H7 (Branger *et al.,* 2005).

All the isolates predictably showed high sensitivity to norfloxacin and ciprofloxacin – the fluoroquinolones. The fluorinated quinolones such as norfloxacin and ciprofloxacin are exceptionally potent against bacteria because their action is at several sites within the *gyrA* and / or topoisomerase IV genes (Ozeki *el al*., 1997). Sensitivity to chloramphenicol was observed in all the PCR confirmed isolates and may be due to the fact that it is not recommended for used in food animals. Chloramphenicol is unavailable in a suitable preparation for use in veterinary practice. This finding is supported by Kabir *et al.* (2002) and Ramon – Yusuf *et al.* (1990) who have reported that resistance to antimicrobial agents is strongly linked to abuse/misuse in animal health and production in Zaria, Northwestern Nigeria. Sensitivity to chloramphenicol and fluoroquinolones is an

important finding since they are common prescription drugs in human medicine (Mora *et al.,* 2005). The emergence and widespread resistance to several antimicrobial agents by bovine isolates of *Escherichia coli* O157:H7 and Shiga toxin-producing *E. coli* O157: H- may have potential negative clinical implications.

Thus continued surveillance of emerging antimicrobial resistance among zonotic food-borne pathogens including STEC is required to ensure public health.

CHAPTER SIX

6.0 Summary, Conclusions and Recommendations

The objectives of this study have largely been achieved. Isolates of *Escherichia coli* O157:H7 from cattle were confirmed as EHEC STEC *E. coli* O157:H7 by PCR assays. The prevalence and distribution of *E. coli* O157:H7 from cattle was reported. The antimicrobial resistance encountered indicate that the extensive use of drugs in animal production and health may have created a pool of resistant *E. coli* O157:H7 and O157: H- that are of public health significance. The resistance patterns obtained suggest involvement of extended spectrum beta-lactameses (ESBLs) and integrons in the acquisition and dissemination of antimicrobial resistance in Northwestern Nigeria

Dairy cattle and nomadic white Fulani cattle were found to harbour *E. coli* O157:H7 and O157: H- in Northwestern Nigeria, and thus may serve as reservoirs. From information available, this study has reported the identification of the s*tx2d* gene in cattle and the occurrence of *E. coli* O157:H7 and O157: H- in cattle in Northwestern Nigeria for the first time.

The recommendations of the study are:

- To undertake molecular characterization of *E. coli* O157:H7 and none H7 strains isolated from cattle and milk in other states/ regions of Nigeria.
- To sample other species of animals, food and humans for the detection of *rfbE, fliC, stx1* and *stx2* genes of *E. coli* O157:H7.

- That more extensive surveys using specific molecular methods in animals, food and man are required to elucidate the epidemiology of this food borne pathogen in Nigeria.
- To confirm the s*tx1*, s*tx2d*, r*fbE* and f*liC* by sequencing of their genes.
- To elucidate the mechanisms of resistance of the isolates to the antimicrobial agents used in the study.

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APPENDICES

APPENDIX 1: LIST OF FARMS SAMPLED

Farm A. Dairy Unit Farm, National Animal Production Research Institute (NAPRI), Shika-Zaria.

Farm B. Jamil Farms, Kaduna State.

Farm C. Niyya Farms, Kaduna State.

Farm D. Rio Hondo Farms, Kaduna State.

Farm E. SMTA Farms, Sokoto State.

Farm F. Yahaya Abdulkarim Farms, Sokoto State.

Farm G. Beef Unit Farm, National Animal Production Research Institute (NAPRI), Shika-Zaria.

Farm H. Professor Esievo Farm, Ahmadu Bello University, Zaria.

Farm I. Professor Gefu Farm, Ahmadu Bello University, Zaria.

APPENDIX 2: PROTOTYPE LETTER OF INTRODUCTION

_____

_____

_____

_____

Dear Sir,

LETTER OF INTRODUCTION FOR DR. IGBAKURA I. LUGA

Dr. Luga is a member of Staff and a Ph.D. Student in this Department. He is carrying out a study on *Escherichia coli*, a major cause of diarrhea in cattle.

Kindly allow him to collect faecal, and feed samples from your farm.

The result of this study should lead to a clearer understanding of *E. coli* infections in cattle and provide recommendations for the prevention of *E. coli* infections and diarrhea in cattle especially calves.

Thanks

Yours faithfully,

Signed:
H.O.D

APPENDIX 3 : PROTOTYPE QUESTIONNAIRE

Dear Sir/Madam,

This is a study of diarrhea in cattle. It has been initiated from Ahmadu Bello University Zaria. The result, it is hoped should aid us towards a better understanding of diarrhea in cattle, leading to its prevention and control.

Dr. I.L.I. Luga

Faculty of Veterinary Medicine
Department Veterinary Public Health and Preventive Medicine
Ahmadu Bello University, Zaria.

Please fill in or tick as appropriate

1. Animals Ear Tag No. and/or name _____
2. Age _____
3. Sex _____
4. Breed _____
5. Location _____
6. Management type_____
7. Presence of diarrhea

   a. Yes ( ) b. No ( )

8. When did the diarrhea start?

   a. 1 day ago ( ) b. 2 days ago ( )
   c. 3 days ago ( ) d. 4 days ago ( )
   e. 5 or more days ago ( )

9. Nature of faeces

   a. Watery ( ) b. Bloody ( )
   c. Mucoid ( ) d. Normal ( )
   e. Others (please specify) _____

10. Other problems noticed

a. Cough ( ) b. Catarrh ( )

c. Others (pleased specify) _____

11. Antibiotic or antiparasitic treatment

a. Yes ( ) b. No ( )

12. If Yes, list the drugs used

a. _____

b. _____

c. _____

d. _____

e. _____

13. When was the treatment administered?

a. 1 day ago ( ) b. 2 days ago ( )

c. 3 days ago ( ) d. 4 days ago ( )

e. 5 or more days ago ( )

14. Number of animals in the herd _____

15. Number currently with diarrhea _____

16. Has there being a diarrhea problem in the herd before?

a. Yes ( ) b. No ( )

17. If yes, when? _____

18. Source of water for the animals (Tick all sources the animals have ever had)

a. Stream ( ) b. Pond lake or dam ( )

c. Well ( ) d. Rain ( )

e. Tap ( )

f. Others (please specify) _____

APPENDIX 4: ENRICHMENT MEDIUM

(Modified Tryptone Soya Broth (MTSB) (Oxoid, Basingstoke, England).

Typical formula (g/l)

Pancreatic digest of casein	17.0;
Peptic digest of soybean meal	3.0;
Sodium chloride	5.0;
Dipotassium hydrogen phosphate	4.0;
Glucose,	2.5;
Bile salts	1.5.

Directions

Add 16.5g to 500ml of distilled water, mix well, and sterilize by autoclaving at 121^{o}C for 15min. Cool to approximately 50^{o}C and aseptically add one vial of novobiocin supplement (Oxoid, Basingstoke, England). Mix well and dispense into sterile containers.

APPENDIX 5: CEFIXIME-SORBITOL–MACCONKEY (CR-SMAC) AGAR

(Oxoid, Basingstake England).

Typical formula (g/l)

Peptone	20.0
Sorbitol	10.0
Bile salts No. 3	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001

Directions

Add 51.5g to 1000ml of distilled water, mix on a hot plate using a magnetic stirrer and bring to boiling point. Sterilize by autoclaving at 121^{o}C for 15 min. Allow to cool to approximately 50^{o}C. Add two vials of reconstituted cefixime selective supplements (Oxoid Basingstoke, England) and dispense into sterile petri disbes (20 ml each).

APPENDIX 6: EOSIN METHYLENE BLUE (EMB) Agar (Oxoid, Basingstoke England)

Typical formula (g/l)

Peptone	10.0
Lactose	10.0
Di-potassium hydrogen phosphate	2.0
Eosin Y	2.0
Methylene blue	0.06
Agar	15.0

Directions

Suspend 37.5g in 7 litre of distilled water, and bring to boil to dissolve completely. Sterilize by autoclaving at 121oC for 15 min. Cool to 60oC and shake the medium well. Dispense 20 ml each into potri dishes and allow to cool and solidify.

APPENDIX 7: TRIPLE SUGAR IRON (TSI) AGAR (Oxoid, Basingstoke England)

Formula (g/l)

Lab-Lemco Powder (Oxoid L29)	3.0
Yeast Extract (Oxoid L20)	3.0
Peptone (Oxoid L37	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferric citrate	0.3
Sodium thisosulphate	0.3
Phenol red	q.s
Agar No. 3 (Oxoid L13)	12.0

Direction

Suspend 65g in 1 litre, bring to boil and dispense into suitable tubes. Sterilize by autoclaving at 121^{o}C for 15 min. Allow the medium to cool in sloped form with a butt about 2.5cm long.

APPENDIX 8: SIMMONS CITRATE AGAR (Oxoid, Basingstoke England)

Formula (g/l)

Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium Ammonium phosphate	0.8
Sodium citrate tribasic	2.0
Sodium chloride	5.0
Bromo-thymol blue	0.08g
Agar No. 3 (Oxoid L13)	15.0

Directions

Suspend 23g in 1 litre of distilled water. Bring to boil. Sterilize by autoclaving at 121^{o}C for 15 min the dispensed 5ml aliquots in test tubes Slant the tubes after autoclaving to cool and solidify.

APPENDIX 9: UREA AGAR BASE (Oxoid, Basingstoke England)

Formulation (g/l)

Peptone	1.0
Glucose	1.0
Sodium chloride	5.0
Disodium phosphate	1.2
Potassium dihydrogen phosphate	0.8
Phenol red	0.012
Agar No. 1	12.0

Directions

Suspend 2.4g in 95 ml distilled water. Allow to soak for 10 min. Stir to mix with a magnetic device at low heat. Sterilize by autoclaving at 121^{o}C for 15 min. Allow to cool to 50^{o}C. Add 5 ml sterile urea solution (Urea 4g, KH_2PO_4, 0.2M 50ml, NaOH, 0.2 N 35ml, Distilled water, ammonia free 115 ml) or 40% urea solution (Oxoid SR20) . Distribute into sterile tubes and allow to set in the sloped position.

APPENDIX 10: SULPHIDE INDOLE MOTILITY (SIM) MEDIUM (Oxoid, Basingstoke England)

Formula (per litre)

Tryptone (Oxoid L42)	20.0g
Peptone (Oxoid L49)	6.1g
Ferrous ammonium sulphate	0.2g
Sodium thiosulphate	0.2g
Agar No. 1 (Oxoid L11)	3.5g

Suspend 30g in 1 litre and bring to boil. Dispense into test tubes and sterilize by autoclaving at 121^{o}C for 15 min.

Indole test: Add one to two drops of Kovac's Reagent (Amyl or isoamyl alcohol 150ml, p-Dimethyl-amminobenzaldehyde 10g, Concentrated hydrochloric acid (HCl) 50ml) and observe the colour change.

Motility: Observe the growth and migration of the organism away from the line of inoculation.

Sulphide test: Black colouration of the SIM medium on inoculation of the organism and incubation at 37^{o}C for 24 hr.

APPENDIX 11: METHYL RED AND VOGES – PROSKAUER MEDIUM (Oxoid, Basingstoke England)

Typical formula (g/l)

Glucose	7.0
Phosphate buffer	5.0’

Suspend 17g in 1 litre of distilled water, mix and distribute into final containers (10ml each) sterilize by autoclaving for 15min at 121^{o}C.

Innoculate the medium and incubate for 24 hr. at 37^{o}C. Divide into two tubes (5ml each).

Methyl red test: Add 5 drops of the methyl red indicator (Methyl red 0.1g, Ethanol 300 ml, Dislilled water 200ml) and read immidietly for colour changes to red

Voges-Proskauer test: Add 1ml of 40% potassium hydroxide and 3ml of 5% α-naphthol in absolute ethanol. Read after 5 min. (Caution: α-naphthol is carcinogenic).

APPENDIX 12: SUGAR FERMENTATION

Sugars

Arabinose

Lactose

Maltose

Mannitol

Sorbitol

Glucose

Directions

Solution I (0.1M NaOH)

Dissolve 4g NaOH in 1 litre of distilled water.

Solution II (Bromothymol Blue Indicator)

Dissolve 0.1g bromothymol blue powder in 2.5ml 0.1M NaOH in a conical flask.

Solution III (Peptone Water).

Dissolve 5g NaCl and 10g of Peptone powder into 1 litre of distilled water and sterilized by autoclaving for 15 min. at 121^{o}C.

Solution IV (10% sugars)

Dissolve each sugar (2.5g) into 25ml of distilled water and sterilize by steaming for 30 min.

Mix 47.5ml peptone water, 0.63 ml bromothymol blue indicator and 25ml for each sugar and dispense into sterile tubes (3ml each) and 5ml for glucose in tubes containing inverted Durham tubes.

Reading Results.

Sugar	**Initial colour**	**Final colour**	**Result**
Arabinose	Blue-Green	Green	-v
		Yellow	+ve
Lactose	Blue-Green	Blue-Green	-v
		Yellow	+ve
Mannitol	Blue-Green	Blue-Green	-v
		Yellow	+ve
Maltose	Blue-Green	Blue-Green	-v
		Yellow	+ve
Srobitol	Blue-Green	Blue-Green	-ve
		Yellow	+ve
Glucose	Blue-Green +	Gas in	+
	Durham tubes	Durham	
		tubes	
		No. Gas	-

APENDIX 13: BRAIN HEART INFUSION (BHI) AGAR (Oxoid)

Typical formula (g/l)

Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Sodium chloride	5.0
Glucose	2.0
Di-sodium phosphate	2.5
Agar	10.0

Directions

Suspend 47g in 1 litre of distilled water. Boil to dissolve. Sterilize by autoclaving at 120^{o}C for 15 minutes. Allow to cool to approximately 50^{o}C and add 7ml of freshly collected human blood for every 100 ml of the suspension. Mix and dispense into sterile petri dishes.

APPENDIX 14: VTEC-RPLA TEST

Assay method

The latex reagents and diluent are ready for use. The latex reagents should be thoroughly shaken before use to ensure an homogeneous suspension. To reconstitute the control toxins, 0.5ml of diluent is added to each vial. The contents are shaken gently until dissolved.

The plate is arranged so that there are 3 columns, each consisting of 8 wells.

Using a pipette or dropper, 25µl of diluent is dispensed into each well.

25µl of test sample supernatant is added to the first well of each column.

Starting at the first well of each column, a pipette or diluter is used to pick up 25µl and perform doubling dilutions down each column, up to and including row 7. (NB: 25µl of sample and Buffer mix must be removed from the 7th well and discarded). The last row of wells is left containing diluent only.

25µl of the test latex VT1 is added to each well in the first column.

25µl of the test latex VT2 is added to each well in the second column.

25µl of latex control is added to each well in the third column for the purpose of detecting false agglutination reactions.

Toxin controls are assayed in the same manner as test samples.

The contents of each well are mixed either by rotating the plate using a micromixer or aggitating by hand. Care must be taken to avoid spillage.

To avoid evaporation, the plate is covered with a lid. (Alternatively, it may be placed in a moisture box). The plate is left undisturbed on a vibration-free surface at

room temperature for 20-24 hours. To assist subsequent reading of the test, the plate may be placed on black paper for the incubation period.

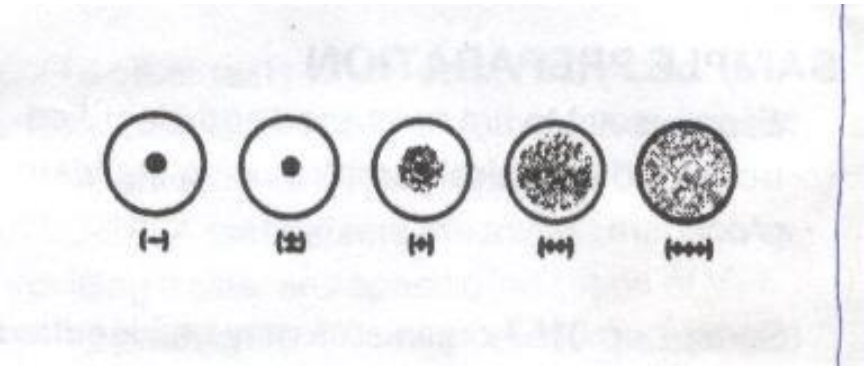
Each well in each column is examined for agglutination against a black background.

Centrifuge tubes, membrane filters, microtitre plates, lids and pipette tips should be sterilized by autoclaving at 121^{o}C or disinfected before disposal in hypochlorite solutions (>1.3% w/w).

Culture extracts, food extracts, samples and toxin controls should be disposed of in hypochlorite solutions (>1.3% w/w).

Interpretation of Test Results

The agglutination pattern should be judged by comparison with the following illustration;



Results classified as (+), (++) and (+++) are considered to be positive. Results classified as (±) and (-) are considered to be negative. Results in the 3 rd column of wells containing latex control should be negative as should the 8th row of columns 1 and 2 which contain no sample. If positive patterns are observed in these wells, the test should be regarded as invalid.

APPENDIX 15: REMEL WELLCOLEX *E. coli* O157LH7 TEST

O157 Procedure

Step 1	Shake the O157 latex reagents	
Step 2	For each test sample place 40µ of saline in two circles on a reaction Card.	40 µl saline
Step 3	Using a mixing stick, remove sufficient growth to just cover the blunt end of the stick. NOTE: The latex is very sensitive, do not pick an excessive amount of culture.	
Step 4	Emulsify the sample of culture in the saline by rubbing with the flat end of the stick. Mix thoroughly, but not too vigorously, or the surface of the card may be damaged, some cultures may be difficult to emulsify. Culture can make the latex appear 'bitty' or 'stringy' on reading. Discard the mixing stick for safe disposal.	Emulsify sample
Step 5	Using a separate stick, emulsify a similar amount from the culture into the saline in the other circle. Discard the mixing stick for safe disposal. Using a separate stick, emulsify a similar amount of sample from the culture into the saline in the other circle. Discard the mixing stick for safe disposal.	Emulsify sample
Step 6	For each test sample place one drop of O157 **Test Latex** in one circle (with the emulsified culture) and one drop of	1 drop

	O157 **Control Latex** in the other circle with the emulsified culture. Ensure that the dropper bottles are held vertically to dispense an accurate drop.	
Step 7	Mix the contents of the circles, carefully spreading the latex over the entire area of the circle. Discard the mixing sticks for safe disposal.	
Step 8	Rock the card slowly for 30 seconds and observe for agglutination. The card should be held at normal reading distance (25 to 35cm) from the eyes. Do not use a magnifying lens.	30 seconds
Step 9	Discard the used Reaction card for safe disposal.	

Cultures that are positive with the O157 Test Latex should be grown overnight at 37^{o}C on blood agar or in tryptone soya broth (TSB) and tested for H7 antigen. Do not test for H7 antigen on cultures grown on SMAC.

H7 Procedure (Blood agar)

Step 1	Shake the O157 latex reagents	
Step 2	For each test sample place 40µl of saline in two circles on a Reaction Card.	40 µl saline
Step 3	Using a mixing stick, remove sufficient growth to just cover the blunt end of the stick. NOTE: The latex is very sensitive, do not pick an excessive amount of culture.	
Step 4	Emulsify the sample of culture in the saline by rubbing with the flat end of the stick. Mix thoroughly, but not too vigorously, or the surface of the card may be damaged, some cultures may be difficult to emulsify. Culture can make the latex appear 'bitty' or 'stringy' on reading. Discard the mixing stick for safe disposal.	Emulsify sample
Step 5	Using a separate stick, emulsify a similar among of same from the culture into the saline in the other circle. Discard the mixing stick for safe disposal. Using a separate stick, emulsify a similar amount of sample from the culture into the saline in the other circle. Discard the mixing stick for safe disposal.	Emulsify sample
Step 6	For each test sample place one drop of **H7 Test Latex** in one circle (with the emulsified culture) and one drop of **H7Control Latex** in the other circle with the emulsified	1 drop

	culture. Ensure that the dropper bottles are held vertically to dispense an accurate drop.	
Step 7	Mix the contents of the circles, carefully spreading the latex over the entire area of the circle. Discard the mixing sticks for safe disposal.	
Step 8	Rock the card slowly for 30 seconds and observe for agglutination. The card should be held at normal reading distance (25 to 35cm) from the eyes. Do not use a magnifying lens.	30 seconds
Step 9	Discard the used Reaction card for safe disposal.	

H7 Procedure (TSB)

Step 1	Shake the O157 latex reagents	
Step 2	For each test sample place 40µl of TSB-grow culture in two circles on a Reaction Card.	40 µl saline
Step 3	For each test sample place one drop of H7 test Latex in one circle (with the broth culture) and one drop of H7 Control Latex in the other circle with the broth culture. Ensure that the dropper bottles are held vertically to dispense an accurate drop.	1drop
Step 4	Mix the contents of the circles, carefully spreading the latex over the entire area of the circle. Discard the mixing sticks for safe disposal.	

Step 5	Rock the card slowly for 30 seconds and observe for agglutination. The card should be held at normal reading distance (25 to 35cm) from the eyes. Do not use a magnifying lens.	3- second
Step 6	Discard the used Reaction card for safe disposal.	

Results

Reading of Results

A positive result is indicated by the development of an agglutinated pattern showing clearly visible clumping of the latex particles. The speed and appearance of the agglutination is dependent on the quality and quantity of the culture antigen.

In a negative result the latex does not agglutinate and the appearance of the suspension remains substantially unchanged throughout the period of rotation. Note: that faint traces of granularity may be detected in the negative results depending on the visual acuity of the operator. In a non-specific result, agglutination occurs in both the Test and Control latexes.

Visual inspection

The latex suspensions should always be inspected for aggregation as they are dropped onto the Reaction Card. If there is evidence of clumping before addition of the test sample the latex suspension should not be used. After prolonged storage some aggregation or drying may have occurred around the top of the bottle. If this is observed the bottle should be shaken vigorously for a few seconds until re-suspension is complete.

Control procedure

When testing clinical specimens, the performance of the test kit reagents should be evaluated by testing both the Positive Control and a Negative Control included in eac test kit. A Positive Control and a Negative Control should be included in each test run. Ensure each are mixed thoroughly before use.

Step 1	Place one drop of each Test Latex in separate circles on a Reaction Card.	1drop
Step 2	Dispense one drop of Negative Control next to each Test Latex.	1 drop
Step 3	Mix using a mixing stick. Discard the mixing stick for safe disposal.	
Step 4	Rock the card slowly for 30 seconds. After this time, definite agglutination should be visible in the Test Latexes.	30 second

Negative Control Procedure

Step 1	Place one drop of each Test Latex in separate circles on a Reaction Card.	1drop
Step 2	Dispense one drop of Negative Control next to each Test Latex.	1 drop
Step 3	Mix using a mixing stick. Discard the mixing stick for safe disposal.	
Step 4	Rock the card slowly for 30 seconds. After this time, definite agglutination should be visible in the Test Latexes.	30 second

The performance of the Test and Control Latex reagents was confirmed using fresh, overnight cultures of reference strains of bacteria, following the method described in Test procedure.

STRAIN	**RESULT**	
	Text Latex (O157 & H7)	**Control Latex**
Positive control *E. coli* O157:H7 (EDL933)	+	-
Negative control *E. coli* 014 (*S. typhimurium*)	-	-

Interpretation of Results

Positive Results

Agglutination of the O157 Test Latex accompanied by a lack of agglutination of the Control Latex indicates the presence of O157 antigen in the culture under test. Cultures that give a positive reaction should be identified biochemically as *E. coli*.

When *E. coli* O157 are identified, they should be further tested for H7 antigen and/or verocytotocin production.

Agglutination of the H7 Test Latex accompanied by a lack of agglutination of the Control Latex indicates the presence of H7 antigen in the culture under test.

E. coli O157:H7 or verocytotocin producing isolates should be reported to local health departments for further evaluation and, if necessary, public health action to prevent further cases.

Negative Result

Lack of agglutination in both O157 reagents means that the culture under test is unlikely to be *E. coli* O157. If minor 'aggregation' corresponding to unemulsified bacteria is seen in both latexes this can be considered as a negative reaction provided the majority of the latex is still present as a smooth, unagglutinated suspension.

Lack of agglutination in both H7 reagents indicates that the culture under test is unlikely to be *E coli* O157:H7, however cultures differ in their expression of H7 antigen and before a negative H7 result can be confirmed the culture must be passaged through motility media and retested.

Non-Interpretable Result

Visible agglutination of the Control Latex indicates a non-specific reaction.