OCCURRENCE AND ANTIBIOGRAM OF ESCHERICHIA COLI O157:H7 IN LOCALLY-FERMENTED MILK (*NONO*) SOLD UNDER MARKET CONDITIONS IN NASARAWA STATE, NIGERIA.

BY

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A THESIS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE

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DECLARATION

I hereby declare that the work in this thesis titled "OCCURRENCE AND ANTIBIOGRAM OF ESCHERICHIA COLI O157:H7 IN LOCALLY-FERMENTED MILK (*NONO*) SOLD UNDER MARKET CONDITIONS IN NASARAWA STATE, NIGERIA" was performed by me in the Department of Veterinary Public Health and Preventive Medicine, under the supervision of Dr. E.C. Okolocha and Dr. M. Bello. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at any institution.

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CERTIFICATION

This thesis, entitled "OCCURRENCE AND ANTIBIOGRAM OF ESCHERICHIA COLI O157:H7 IN LOCALLY-FERMENTED MILK (*NONO*) SOLD UNDER MARKET CONDITIONS IN NASARAWA STATE, NIGERIA" by Christopher Rine Reuben meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I wholeheartedly dedicate this piece of work to the all sovereign God, the wonderful counsellor, the great teacher and the giver of true knowledge, wisdom and discernment.

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Grace, mercy and peace be multiplied daily to you in Jesus name (Amen).

Abstract

Escherichia coli O157:H7 is a newly emerging pathogen frequently associated with the consumption of food of bovine origin. Severe and life threatening human diseases caused by *E. coli* O157:H7 strains have been reported throughout the world. The present study evaluated the occurrence of *E. coli* O157:H7 in locally fermented milk (*nono*) sold under market conditions in Nasarawa State, Nigeria and the patterns of their antibiotic susceptibility. A total of 420 *nono* samples were purchased across Nasarawa State. The samples were bacteriologically analyzed in the laboratory for the presence of *E. coli* O157:H7 by means of cultural techniques (involving enrichment on modified tryptone soy broth and selective plating on Cefixime-Tellurite Sorbitol McConkey Agar), biochemical (Microbact 12E) and serological assays. Oxoid diagnostic kit, latex (R30959601) was used to confirm *E. coli* O157:H7. Confirmed isolates were further subjected to antimicrobial susceptibility test using the agar disc diffusion technique. The results of the study showed that out of 420 *nono* samples examined, 19 (4.5%) were contaminated with *E. coli* O157:H7. The highest occurrence rate (5.7%) was recorded in samples obtained from Akwanga, Wamba and Doma Local Government Areas, while Lafia and Keffi had the least occurrence rate (2.9%). With respect to the senatorial zones, Nasarawa North had the highest occurrence rate of 5.7% while the Southern zone had the least (3.6%). There was no significant difference ($P>0.05$) in the occurrence of *E. coli* O157:H7 isolated from *nono* samples with respect to the various Local Government Areas. Antibiotic susceptibility profiles showed that all the isolates were resistant to multiple antibiotics, except ciprofloxacin and gentamicin, resulting in nine different resistant patterns. All the 19 (100%) isolates were resistant to penicillin and tetracycline, 18 (94.7%) to erythromycin, 16 (84.2%) to amoxicillin, oxacillin and

sulphamethoxazole/ trimethoprim, 13 (68.4%) to chloramphenicol and 8 (42.1%) to streptomycin; 15 (78.9%) and 17 (89.5%) of the isolates were sensitive to ciprofloxacin and gentamicin respectively. The predominant antimicrobial resistance pattern was penicillin-tetracycline-chloramphenicol-amoxicilin-erythromycin-oxacillin-sulphamethoxazole/ trimethoprim with the occurrence rate of 36.8% among the 19 isolates tested. *Nono* consumption has potential health risks to consumers not just in Nasarawa State but possibly to the nation at large. Hence proper hygiene in the processing and marketing of *nono* is recommended. The multiple antimicrobial resistance exhibited by *E. coli* O157:H7 strains in this study is an indication of possible antibiotic abuse.

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
E. coli	*Escherichia coli*
LPS	Lipopolysaccharide
PCR	Polymerase chain reaction
Stx	Shiga toxin
*eae*A	Intimin gene
*hly*A	Haemolysin gene
RAPD	Random amplified polymorphic DNA
EHEC	Enterohaemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
VTEC	Verocytotoxigenic *E. coli*
HC	Haemorrhagic colitis
HUS	Haemolytic uraemic syndrome
TTP	Thrombotic thrombocytopenic purpura
VT	Verotoxin
Ehly	Enterohemolysin
mTSB	Modified tryptone soya broth
SMA	Sorbitol MacConkey Agar
NSF	Non sorbitol fermenter
SF	Sorbitol fermenter
CT- SMA	Cefixime-Tellurite Sorbitol McConkey Agar

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Nono is a Nigerian locally fermented milk product commonly prepared by the Hausa/Fulani cattle rearers. It is mostly available in the northern and central parts of Nigeria (Bankole, 1990; Yahuza, 2000). It is a healthy food whose consumption traverses the Saharan tribes of the West African sub region extending to the inhabitants of the Mediterranean region and also the Middle East (Uzeh *et al.*, 2006; Nahar *et al.*, 2007; Ogbonna, 2011).

Nono is produced in homes, especially in villages where the shelf life and safety of the product is not considered. It is however sold to both rural and urban people in combination with "fura" as food (Uzeh *et al*., 2006).

Nono is produced from non-pasteurized cow milk collected in a calabash and allowed to ferment naturally for 24 hours (Ekah *et al*., 1977; Olasupo *et al.*, 1996). *Nono* also called *nunu* by some tribes in Nigeria contains high quantities of amino acids, calcium, phosphorus, and vitamins A, C, E and the B complex (Nebedum and Obiakor, 2007).

It was noticed that raw milk often contains microorganisms which may likely cause food borne diseases (Adesiyun, 1995; Headrict *et al*., 1998). Even when the milk is fermented, the fermentation process with the attendant drop in pH may not rid the product of these organisms and may be carried to consumers (Ogbonna, 2011).

Raw or fermented milk is a well known medium that supports the growth of several kinds of microbes with resultant spoilage of the product and/or infections/intoxications in consumers

(Murinda *et al*., 2004; Oliver *et al*., 2005). *Escherichia coli* frequently contaminate milk and milk products and it is an established indicator of faecal or environmental contamination (Soomro *et al*., 2002; Benkerruin *et al*., 2004). *E. coli* may gain entry into raw milk directly from dairy cows experiencing sub-clinical or clinical mastitis (Rodojcic-Prodaova *et al*., 1991), from the farm environment particularly the water source (Eberhart, 1977) and utensils used for the storage of milk on farm or during transportation (Freedman, 1977).

E. coli is the species most commonly isolated from human faecal samples and is part of the normal intestinal flora of healthy individuals. *E. coli* O157:H7 strain is the classical serotype linked to serious outbreaks and sporadic cases of enterohaemorrhagic diseases such as haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) (Muller and Ehlers, 2005; Mashood *et al*., 2006). Cattle are the main reservoir for *E. coli* O157:H7, but the bacteria also occurs in other animal species such as sheep, goats, pigs, cats, dogs, chickens and gulls (Callaway *et al*., 2003; Muller and Ehlers, 2005; Mashood, *et al*., 2006).

E. coli O157:H7 is one of the most common agents of food borne illness in humans (Buzby *et al*., 1996). *E. coli* O157:H7 has been isolated from beef and dairy cattle at all stages of production, and although, their shedding are intermittent and can be difficult to detect, this *E. coli* serotype appears to be fairly widespread throughout bovine population (Fedorka-Cray *et al*., 1998). Markets and consumers for raw milk and milk products have existed in many parts of the world (Asmahan and Warda, 2011).

1.2 Statement of the Research Problem

One of the most significant food-borne pathogens that have gained increased attention in recent years is *E. coli* O157:H7. *E. coli* O157:H7 was first recognized as food pathogen in 1982 (Riley

et al., 1983). Since then, it has become a pathogen of major concern in both food and dairy industries, and to the public, because of its ability to cause severe illness, in particular, haemorrhagic colitis, haemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Abdul-Raouf, 1993). Domestic and wild animals are the sources of *E. coli* O157:H7, but ruminants are regarded as the natural reservoir. Sporadic cases and outbreaks of human diseases caused by *E. coli* O157:H7 have been linked to ground beef, raw milk, meat and dairy products, vegetables, unpasteurized fruit juices and water (Chapman *et al*., 1997). Infection can also be acquired by direct contact with animals and by person-to-person spread (Cho *et al*., 2006). In different countries, people have suffered from many infections caused by *E. coli* O157:H7. This bacterium has been found in healthy bovine faeces. Therefore, milk and milk products produced from bovine may pose a risk of infection if the milk is not adequately handled (Arici *et al*., 2004).

Although, antibiotics are not recommended for treatment of *E. coli* O157:H7 infections in humans, there is evidence that bacterial isolates are resistant to some antibiotics (Aibinu *et al*., 2007). Smith *et al*. (2003) reported multidrug resistance in isolates of *E. coli* O157:H7 strains obtained from farm-animals and human infections in Lagos and Ogun States in Nigeria. They had collected a total of 350 fresh faecal droppings of animals (cattle, pig, chicken and sheep) and human stool samples comprising of diarrhoeic (150) and non diarrhoeic (50). Carl *et al*., (2002) reported that 361 *E. coli* O157:H7 isolates kept at the *E. coli* Reference Centre at the Pennsylvania State University were resistant to sulphamethoxazole (26%), tetracycline (27%), cephalothin (17%) and ampicilin (13%). These isolates were recovered from samples collected from humans, cattle, swine, and food for a period spanning the years 1985 to 2000. The extensive use of antibiotics in both human medicine and for agricultural purposes, particularly, in

disease prevention and growth promotion in animal production is a considerable cause of the selection and prevalence of antibiotic resistant *E. coli* O157:H7 (Schroeder *et al*., 2002; Callaway *et al*., 2003). The development of resistance to antimicrobials is known to occur through stable genetic change heritable from generation to generation through specific mechanisms including mutation, transduction, transformation and or conjugation (Goodman *et al*., 1990).

Because some antibiotics may cause bacterial lysis and liberate the free Shiga toxins in the intestinal tract (Karch *et al*., 1987; Wong *et al*., 2000), the antimicrobial treatment is contraindicated for human *E. coli* O157:H7 infections. However, such treatments may be recommended for cystitis and pyelonephritis other than haemorrhagic colitis all caused by *E. coli* O157:H7 (Griffin and Tauxe, 1991). For those limitations of using antimicrobial agents in *E. coli* O157:H7 cases, the generally accepted belief is that the *E. coli* O157:H7 may still be susceptible to most antimicrobials. In addition to their epidemiological importance, the studies of antimicrobial susceptibility of *E. coli* O157:H7 may have more therapeutic significance as recent studies have indicated a possible role of early administration of antimicrobials in preventing the progression of haemolytic uremic syndrome and haemorrhagic colitis both caused by *E. coli* O157:H7 (Molbak *et al*., 2002).

1.3 Justification of the Study

E. coli O157:H7 has become an important pathogen with worldwide distribution (Schlundt, 2002). Food borne infection with *E. coli* O157:H7 continues to be a significant public health problem in Nigeria. A great proportion of the milk produced in tropical countries is converted into indigenous products like *ghee* or some kind of fermented or concentrated products that are

kept without artificial cooling (Okolocha *et al*., 2008). Various bacteriological analyses of locally fermented milk (*nono*) revealed various significant prevalence rates of the occurrence of *E. coli*, especially serotype O157:H7 including 18% in Zaria (Asuke *et al*., 2012), 46.6% in Kano (Bukar *et al*., 2010), 8.6% in Ibadan (Adetunji *et al*., 2011), and 43% in Maiduguri (Ogbonna, 2011).

Although, dairy products and especially fermented milk (*nono*) have been implicated in outbreak of *E. coli* O157:H7 (Chapman, 2000), there is little or no documented study to the best of my knowledge on the prevalence of *E. coli* O157:H7 in *nono* sold in Nasarawa State under market conditions. Sequel to the massive movement of Fulani cattle rearers and their cattle into the state from the surrounding states due to insecurity and religious, ethnic and political crises, there is need to conduct such a survey, due to the increase in *nono* production and hawking which has invariably led to its high demand within the state. In developing countries such as Nigeria, there are serious concerns about sanitation of ready-to-eat foods (especially milk and milk products), particularly as potable water is seldom available to processors and also, most food handlers lack basic knowledge of proper personal and environmental hygiene (Bukar *et al*., 2010). With the increase in cattle population, the high number of nomadic and settled herds and established farms, Nasarawa State occupies a critical position in the realization of the goal of producing high animal protein. It is in view of these that this study was conducted.

1.4 Aim of the Study

The aim of this study is to determine the occurrence of *E. coli* O157:H7 in locally fermented milk (*nono*) sold under market conditions in Nasarawa State, Nigeria and to study their antibiotic resistance patterns.

1.5 Objectives of the Study

i. To isolate and determine the prevalence of *E. coli* O157:H7 in *nono* sold under market conditions in Nasarawa State, Nigeria.

ii. To determine the antibiotic resistance patterns of *E. coli* O157:H7 isolates obtain from the study.

1.6 Research Hypotheses

i. *Nono* sold in Nasarawa State under market conditions do not contain *E. coli* O157:H7

ii. *E. coli* O157:H7 isolated from *nono* sold under market conditions in Nasarawa State are not resistant to antibiotics.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Escherichia coli* O157:H7

2.1.1 History

E. coli O157:H7 is a relatively new pathogen. It was first described in 1977 by Konowalchuk, Speirs and Stavric (1977) who found that certain diarrhoeagenic *E. coli* strains produce a cytotoxin that can kill Vero cells, thus they named it verotoxin (VT). In 1982, *E. coli* O157:H7 was recognized for the first time as a human pathogen when two outbreaks occurring in Oregon and Michigan were associated with eating undercooked hamburgers from a fast food restaurant chain (Riley *et al*., 1983). This outbreak was characterized by a distinctive haemorrhagic colitis, and a rare isolate of O157:H7 serotype of *E. coli* was implicated as the agent of disease. Searches of culture collections in the USA dating from 1973, and in Canada and the UK dating from 1978, found only eight *E. coli* O157 isolates deposited before 1982 (Griffin and Tauxe, 1991).

Unfortunately, it was not until 1993, following a large outbreak with more than 700 cases infected by eating contaminated fast food hamburgers, that *E. coli* O157:H7 was recognized as a major food safety issue (Bell *et al.*, 1994). During the past 30 years, an increasing number of *E. coli* O157:H7 outbreaks have gained a worldwide niche as a formidable public-health concern. The US Department of Agriculture (USDA) has established a 'zero tolerance' policy for *E. coli* O157:H7 in ground beef. However, this policy has not resolved the problem, as an estimated 73,480 illnesses due to *E. coli* O157:H7 infections occur in the United States every year, leading to an estimated 2,168 hospitalizations and 61 deaths annually (Mead and Griffin, 1998).

2.1.2 Taxonomy

E. coli is a Gram-negative, non-spore-forming facultative anaerobe in the family of *Enterobacteriaceae*. Serotyping and serogrouping of *E. coli* is used for subdividing the species into serovars. Serotyping in *E. coli* involves serological identification of three surface antigens: O (somatic lipopolysaccharide), K (capsular) and H (flagellar). Orskov and Orskov (1984) estimated that 173 O antigens, 80 K antigens and 56 H antigens existed. Based on the presence of certain virulence factors and their interaction pattern with mammalian cells or tissues and toxin production, pathogenic *E. coli* are also classified in six virotypes (1) enterotoxigenic *E. coli* (ETEC), (2) enteropathogenic *E. coli* (EPEC), (3) enterohaemorrhagic *E. coli* (EHEC), (4) enteroinvasive *E. coli* (EIEC), (5) enteroaggregative *E. coli* (EAEC), and (6) diffusely adhering *E. coli* (DAEC) (Bhunia, 2008). The first documented and most well studied EHEC strain is *E. coli* O157: H7 which is also considered the prototypical serotype of EHEC.

2.1.3 Biology/ Toxins

Most *E. coli* are generally considered to be harmless as members of the indigenous microbiota in the gut of humans and warm-blooded animals; however, there are also some serotypes that are responsible for causing severe foodborne disease such as bloody diarrhoea and haemolytic uremic syndrome (HUS), and the disease is prevalent in developed countries such as the USA, Japan and the United Kingdom. As opposed to other commensal strains, *E. coli* O157: H7 generally does not ferment sorbitol and does not have β-glucuronidase activity, which is a very useful marker for bacterial identification. It grows rapidly at 30 – 42^0C, does not grow at 10 ^{0}C or below and does not grow or grows poorly at 44^0C or above with an optimum temperature of 37^0C (Bhunia, 2008). The organism is destroyed by thorough cooking of foods when all parts reach a temperature of 70^0C or higher, however, it can survive for weeks at 4^0C or even -20^0C. Although there is some variability among strains, this pathogen is relatively acid-tolerant and can grow at pH levels ranging from 4.4 – 9.0, a_w of > 0.95 and NaCl levels of < 8.5%. In foods at pH levels of 3.5 – 5.5, it can survive for extended periods (Riemann and Cliver, 2006). *E. coli* O 157:H7 can produce two different Shiga-like toxins, namely SLT1 and SLT2. These Shiga-like toxins are very similar, if not identical, to the toxins produced by *Shigella dysenteriae*. Due to their toxic effects on Vero (African green monkey kidney) cells, *E. coli* O157:H7 is also known as verotoxin-producing *E. coli* (VTEC). The growth of this pathogen in the human body produces a large quantity of these toxins that cause severe damage to the lining of the intestine and may cause additional damage to kidneys, pancreas and brain. In addition to Shiga-like toxins, *E. coli* O157:H7 can produce other virulence factors that may increase the severity of human illnesses. These virulence factors include intimin and enterohaemolysin, which are responsible for intimate attachment to the intestinal surface and enterocyte damage, respectively (Riemann and Cliver, 2006).

2.2 Outbreaks of *E. coli* O157:H7

Following identification of *E. coli* O157:H7 as the causative agent of human illness in 1982 (Riley *et al*., 1983), numerous outbreaks and sporadic cases of illness associated with *E. coli* O157:H7 have been reported from Argentina, Australia, Belgium, Canada, China, Czechoslovakia, England and Wales, Ireland, Italy, Japan, Korea, Mexico, New Zealand, Romania, Scotland, Spain, Thailand and the United States (Griffin and Tauxe 1991; Besser *et al*., 1993; Chapman, 1995; Ackman *et al*., 1997; Keene *et al*., 1997; Swerdlow and Griffin 1997; Cody *et al*., 1999; Michino *et al*., 1999). Increased recognition of *E. coli* O157:H7 as a cause of human illness has resulted in increased surveillance for *E. coli* O157:H7 worldwide. Seemingly more frequent isolation of the organism in developed countries is likely due to more advanced reporting systems and more sensitive technologies for detection.

Most foodborne outbreaks have been traced to foods derived from cattle, especially ground beef and raw milk (Mead *et al*., 1998; Doyle *et al*., 2006). In the 1980s, most outbreaks were associated with inadequately cooked hamburgers and unpasteurized milk (Doyle *et al*., 2006). Later, outbreaks were traced to other dairy products such as yogurt and cheese (Doyle *et al*., 2006). Besides, fruits and vegetables such as lettuce, apple cider, unpasteurized apple juice and alfalfa sprouts have been implicated in *E. coli* O157:H7 outbreaks (Mead *et al*., 1998). These outbreaks may be due to contact with animal faeces at some stage during cultivation or handling (WHO, 2005). Over the years, contaminated water has been reported increasingly as a source of human infection, including drinking water contaminated with animal faeces and swimming in lake and pool water (Doyle *et al*., 2006). Apart from contaminated food and water, person-to-person transmission spread by the faecal oral route was also documented in many outbreaks (Doyle *et al*., 2006).

In the United States, *E. coli* O157: H7 causes over 73,000 illness each year and approximately 61 deaths (Mead *et al*., 1999). The hospitalization rate and case-fatality rate is respectively 0.295 and 0.0083 (Mead *et al*., 1999). In Europe, wide variation was seen in incidence rates between countries (Table 2.1). Lowest incidence rate of 0.1 per million was reported in Spain, while highest incidence rate of 20.3 per million in the United Kingdom. As with isolation rate in cattle and ground beef, seasonal trend is also observed in human infections, with more outbreaks during the summer months (Chapman *et al*., 2000).

Outbreaks of infection, generally associated with beef, have been reported in Australia Canada, Japan, United States, in various European countries, and in southern Africa. In 1996, an outbreak of *E. coli* O157:H7 in Japan affected over 6,300 school children and resulted in 2 deaths. This is the largest outbreak ever recorded for this pathogen (WHO, 2005). Similarly, in 1999 in the United States, *E. coli* O157 outbreaks were reported from 30 states and affected 1897 persons. Two hundred and one (11%) persons were hospitalized, 37 (2%) developed HUS and 4 (0.2%) died (CDC, 1999).

Table 2.1 summarizes the key findings of the large outbreaks (affecting more than 200 persons) reported. The largest *E. coli* O157:H7 outbreak took place in Sakai City of Japan in 1996 in which the radish sprouts in school lunches were contaminated, causing a total of 9451 cases with 12 deaths (Doyle, 2006).

In Asia, other than Japan, relatively few outbreaks of *E. coli* O157:H7 have been reported. In Mainland China, *E. coli* O157:H7 was first identified from patients suffering from haemorrhagic colitis in Jiangsu Province in 1986 (Xu *et al*., 1999). No major outbreaks of *E. coli* O157:H7 have been reported except for one in Jiangsu Province in 1999, which involved 95 *E. coli*

O157:H7 infected patients developing acute renal failure with a case fatality rate of 87% (Wang, 2005). In Taiwan, EHEC is a notifiable disease but as of August 2009, only one confirmed case had been recorded in 2001 (Wang, 2005).

Okeke *et al*. (2000) carried out a study in small-town and rural primary health care centres in South-western Nigeria. A total of 330 *E. coli* strains isolated from 180 children with diarrhoea and from 144 apparently healthy controls were examined for virulence traits. Using the colony blot hybridization, the results showed that 1.2% of the strains were enterohaemorrhagic *E. coli*. The *E. coli* strains that hybridized with a shiga toxin gene probe that lacked other characteristics usually present in enterohaemorrhagic *E. coli* constituted 8.4%.

Table 2.1 Large *E. coli* O157:H7 outbreaks (affecting over 200 persons) worldwide (1982 – 2006) (Doyle, 2006)

Reported year	**Country**	**No. of Persons Affected (death)**	**Vehicle**
1990	US	243 (4)	Well water
1991	Canada	521 (2)	Minced beef and caribou
1992-1993	US	>700 (3)	Hamburger
1995	UK (Scotland)	633 (0)	Sewage contaminated drinking water
1996	Japan(Sakai)	9451 (12)	Radish sprouts
1996	UK(Scotland)	503 (20)	Lunch foods

1996-1997	UK(Scotland)	512 (17)	Meat from a shop
1997	UK	332 (0)	Restaurant food
1999	US	>1,000 (2)	Well water
1999	US	329	Beef
2000	Canada	2,300 (7)	Drinking water
2000	US	788 (1)	Raw beef, cross contamination of other foods
2006	US(26 States) and Canada	204 (3)	Spinach

Olorunshola *et al*. (2000) examined the prevalence of sorbitol-nonfermenting *E. coli* O157:H7 (EHEC) in 100 patients with diarrhoea by stool culture on sorbitol Macconkey agar in Lagos Nigeria. The detection rate of O157:H7 was 6%. Five of the six patients were children below 5 years of age and one was a teenager. Recently, Smith *et al*. (2003) reported 17% EHEC O157:H7 from healthy animals in Lagos Nigeria. However, report shows that there was no isolate of EHEC O157:H7 from patients with diarrhoea in the South-Western part of Nigeria (Okeke *et al*., 2003)

2.3 Reservoirs and Sources of *E. coli* O157:H7

Several reservoirs and sources of *E. coli* O157:H7 have been identified:

2.3.1 Cattle: the primary reservoir

Subsequent to the first two outbreaks of *E. coli* O157:H7 infection in 1982, a bovine source of the pathogen was implicated (Riley *et al.*, 1983; Wells *et al.*, 1983). Early studies indicated that the prevalence of *E. coli* O157:H7 in the faeces of cattle in the United States was low (Hancock *et al.*, 1994; Faith *et al.*, 1996). Hancock *et al.* (1994) reported this organism to be present in dairy and beef cattle at 0.28% and 0.71%, respectively, with herd prevalences of 8.3% and 16%, respectively. More recent studies, however, suggest much higher levels of occurrence. Chapman *et al.* (1997) isolated *E. coli* O157:H7 from the faeces of 15.7% of cattle (752/4800) over a 1-yr period. Monthly prevalences ranged from 4.8 to 36.8%, and were highest in the spring and late summer. Van Donkersgoed *et al.* (1999) isolated *E. coli* O157:H7 from 7.5% of faecal samples collected from cattle at slaughter. In that study, the prevalence of *E. coli* O157:H7 was higher in samples from yearling cattle than from cull cows (12.4 vs. 2.0%). Even more recently, Elder *et al.* (2000) reported *E. coli* O157:H7 to be present in 91 of 327 faecal samples (28%) taken from slaughter cattle in the Midwestern United States during July and August. Another survey in the United States found *E. coli* O157:H7 in 63 of 100 feedlots tested, indicating widespread distribution of this organism in cattle operations. The increasing prevalence of *E. coli* O157:H7 has likely arisen from the use of more sensitive isolation methods. Immunomagnetic separation has increased the sensitivity of detection of *E. coli* O157:H7 by 10- to 100-fold (Chapman *et al.*, 1994).

Repeated isolation of *E. coli* O157:H7 from healthy beef and dairy cattle demonstrates that cattle are asymptomatic carriers of the organism (Wells *et al*., 1999; Hancock *et al*., 1994; Zhao *et al*., 1995). Thus, the presence of the organism in an individual animal or livestock operation cannot be signalled by morbidity. At present, faecal analysis is the only practical means of confirming or enumerating *E. coli* O157:H7. This task is complicated further by the fact that faecal shedding of *E. coli* O157:H7 by cattle is intermittent. Short periods of relatively high prevalence of excretion are separated by longer periods of reduced or undetectable shedding (Wells *et al*., 1999; Besser *et al*., 1997). This situation has undoubtedly contributed to the variance in prevalence reported in the literature.

Among those animals reported to be shedding *E. coli* O157:H7, levels of shedding have ranged from 102 to 105 cfu g^{-1} of faeces. The shedding itself tends to be seasonal, peaking in the summer months (May through September) (Hancock *et al*., 1994; Van Donkersgoed *et al*., 1999). The predominant site of *E. coli* O157:H7 persistence and proliferation in the adult bovine gastrointestinal tract is the colon (Zhao *et al*., 1995; Grauke *et al*., 2002). In humans, the virulence of this pathogen is associated mainly with its VT production. Receptors for these cytotoxins are present primarily in the vasculature of the colon and the kidney (Schmidt *et al*., 1995). In contrast, VT receptors are present in the bovine intestinal tract and kidney, but not in the vasculature, which may enable the asymptomatic carriage of *E. coli* O157:H7 by cattle (Hoey *et al*., 2002).

2.3.2 Other animal sources of *E. coli* O157:H7

Epidemiological investigations of animal populations as reservoirs of *E. coli* O157:H7 have concentrated on bovine breeds but other animal hosts have also been identified (Beutin *et al*., 1995). In the United Kingdom, Chapman *et al*. (1997) reported isolation of *E. coli* O157:H7 from 2.2% of sheep and 0.4% of pigs at slaughter, but the organism was not detected in samples from 1000 chickens. In other studies in the United States, The Netherlands and Australia, the prevalence of *E. coli* O157:H7 in sheep ranged as high as 31%, indicating that sheep may also play a significant role in harbouring *E. coli* O157:H7 (Kudva *et al*., 1996; Heuvelink *et al*., 1998; Fegan and Desmarchelier, 1999). Trepeta *et al*. (1984) suggested that horses and dogs may be vectors of *E. coli* O157:H7, due to the close contact between farm animals, companion animals and humans. Hancock *et al*. (1998) reported detection of *E. coli* O157:H7 in 1.1% (1/90) of horses and 3.1% (2/65) in dogs in the United States. *E. coli* O157:H7 has also been isolated in the United States from wild deer, white-tailed deer and the faeces of wild birds and from birds in the United Kingdom (Rice *et al*., 1996; Keene *et al*., 1997; Hancock *et al*., 1998). The avian isolations suggest the possibility of transfer of *E. coli* O157:H7 between animals sharing the same habitat.

The high variability of prevalence of *E. coli* O157:H7 among cattle suggests the possibility of a reservoir of *E. coli* O157:H7 external to cattle (Hancock *et al*., 1998). The intermittent and seasonal nature of their faecal shedding of *E. coli* O157:H7 could reflect cattle functioning more as a vehicle for transmission of the organism than as a reservoir in which the organism is maintained for long periods of time (Hancock *et al*., 1998). Reports of *E. coli* O157:H7 in other animal species and birds confirm the existence of non-bovine animal reservoirs or vehicles of *E. coli* O157:H7. Other than the confirmation of the presence of *E. coli* O157:H7 in non-bovine species, however, the ecology of *E. coli* O157:H7 beyond cattle has not been extensively studied.

2.3.3 Environmental sources of *E. coli* O157:H7

Johnson *et al*. (1996) outlined many possible habitats for *E. coli* O157:H7 in the farm environment, including manure heaps, ponds, dams and wells, barns, calf hutches, straw and other beddings, feed and feed troughs, water and water troughs, farm equipment, ground and pasture, and water courses, and noted that once present in the environment, this organism can be transferred to other sites by rainwater, wind, and removal and spreading of manure, as well as by animals and humans. Aspects of survivability and transmissibility of *E. coli* O157:H7 in many of these habitats have been investigated.

2.3.3.1 *Feeds*

E. coli O157:H7 was isolated from 6.3% of feed samples obtained from a dairy farm in Wisconsin (Shere *et al*., 1998). Van Donkersgoed *et al*. (1999) isolated *E. coli* O157:H7 from feed in 1.7% of feed bunks in feedlot pens but found that total mixed rations (TMR) did not support the growth of the organism. Conversely, growth of *E. coli* O157:H7 has been demonstrated in a variety of wet grain mixtures and some silage-based mixtures *in vitro* and poorly fermented laboratory silage has also been shown to support the growth of *E. coli* O157:H7 (Lynn *et al*., 1998; Fenlon and Wilson, 2000). Two strains of *E. coli* O157:H7 inoculated at harvest onto grass ensiled under intentionally poor conditions increased from 103 cfu g^{-1} to 107 cfu g^{-1} over 13 d at 20°C. The poor ensiling technique likely delayed the silage fermentation, creating an environment conducive to growth of *E. coli* O157:H7. Faecal contamination of forage (e.g., from manure application) followed by inadequate ensiling may therefore be a significant factor in the transmission and persistence of *E. coli* O157:H7 in the feedlot environment (Fenlon *et al*., 2000).

Adequate ensiling, on the other hand, has been shown to reduce the risk of *E. coli* O157:H7 contamination of forage. In a laboratory-scale mini-silo trial, barley forage was treated at harvest with 105 cfu g^{-1} of O157:H7 and non-O157:H7 strains of *E. coli* and ensiled for 42 days with and without a bacterial silage inoculant designed to accelerate the fermentation and decline of pH in the silage. All the *E. coli* were eliminated within 15 days, including the silage inoculant significantly reduced the persistence of *E. coli* O157:H7. Thus, this strategy would allow producers to feed silage out shortly after ensiling with minimal risk of transmitting *E. coli* O157:H7 to cattle (Bach *et al*., 2002).

The seasonality of faecal shedding of *E. coli* O157:H7 implies that environmental replication may play a key role in its ecology in the farm environment. Given that this organism can replicate in a variety of feeds, thus amplification of low levels of *E. coli* O157:H7 may increase the possibility of further dissemination of the organism on the farm.

2.3.3.2 *Water for livestock*

Water for livestock may become contaminated by bacteria from numerous sources. Cattle may contaminate their troughs with faecal material, feed, bedding or saliva (LeJeune *et al*., 2001). In some cases, water may be contaminated even before it enters the trough (Van Donkersgeod *et al*., 1999).

Several studies have documented isolation of *E. coli* O157:H7 from animal drinking water (Faith *et al*., 1996; Hancock *et al*., 1998; Buchko *et al*., 2000; LeJeune *et al*., 2001b). This organism survived for over 6 months in the sediments of microcosms designed to simulate cattle water troughs, and these contaminated microcosms effected colonization of 10-week old calves with *E. coli* O157:H7. *E. coli* O157:H7 may exist in water in a viable but nonculturable (VBNC) state

for long periods of time, all the while retaining pathogenic potential. *E. coli* has also been shown to form biofilms. Active bacterial growth and sloughing in response to biocides suggested that a protective capacity of biofilms allowed these microorganisms to persist in water distribution systems (Daly *et al*., 1998). Thus, water troughs can serve as environmental reservoirs of *E. coli* O157:H7 and contribute to subsequent infection of cattle (Lejeune *et al*., 2001a).

There is evidence that drinking water for livestock may become contaminated by oral rather than faecal routes. *E. coli* O157:H7 was detected in drinking water obtained from covered water tanks equipped with ball-valved watering ports (Shere *et al*., 1998). Those researchers discounted faecal contamination of that watering system, given that delivery of water required physical depression of the ball in the port by the animal. Recovery of *E. coli* O157:H7 from the tonsils (Cray and Moon, 1995) and saliva (Buchko *et al*., 2000; Keen and Elder, 2002) of cattle further supports the possibility of oral contamination of their drinking water. Moreover, recovery of *E. coli* O157:H7 in the faeces of previously O157-negative cattle subsequent to their having ingested water harbouring that bacterium suggests that drinking water may be a source of infection (Shere *et al*., 1998).

LeJeune *et al*. (2001) isolated *E. coli* O157:H7 from 6 of 473 water troughs located at 99 different cattle operations. The extent of contamination of water troughs with *E. coli* was positively correlated with proximity of the troughs to the feed bunk, protection of the trough from direct sunlight, warmer environmental temperatures and reduced protozoal concentrations in the water troughs. Reducing the protozoal population in an experimental microcosm also resulted in increased populations of *E. coli* O157:H7 (LeJeune *et al*., 2001). These results suggest that many factors that dictate microbiological quality of animal drinking water are controllable.

It is evident that water troughs may serve as long-term reservoirs of *E. coli* O157:H7. Survival of *E. coli* O157:H7 in water for extended periods of time, in addition to the potential of contaminated water as a point source of infection, emphasizes the importance of control of *E. coli* O157:H7 in drinking water for livestock.

2.3.3.3 *Manure*

Reports regarding the relationship between application of manure to grazing land and the prevalence of *E. coli* O157:H7 in cattle have been inconsistent. Hancock *et al*. (1994) reported a tentative association between applying slurry to grazing land and the *E. coli* O157:H7 infection status of the herd. Subsequent studies, however, reported no association between manure application to pastures or forage crops for cattle and *E. coli* O157:H7 faecal shedding by cattle (Garber *et al*., 1995). More recently, a study by Bolton *et al*. (1999) indicated that current waste management practices such as spreading manure on pasture land may increase the carriage rate of *E. coli* O157:H7 in herds.

Survival of *E. coli* O157:H7 in manure and manure slurry has been observed under various experimental and environmental conditions. Kudva *et al*. (1998) found that *E. coli* O157:H7 from experimentally inoculated sheep survived for 21 months in manure exposed to environmental conditions. In aerated piles of bovine and ovine manure, the organism survived for 4 months and 47 days respectively. In bovine faeces inoculated initially with 105 cfu g^{-1}, *E. coli* O157:H7 survived for 70, 56 and 49 days, at 5, 22 and 37°C, respectively (Wang *et al*., 1996). Fukushima *et al*. (1999) found that *E. coli* O157:H7 inoculated at 103 cfu g^{-1} survived in bovine faeces for 2 to 14 weeks at 5°C, 1 to 18 weeks at 15°C, and 3 to 12 weeks at 25°C.

Contamination with bovine manure has been suggested in several cases of human infection associated with non-bovine food products. Inadequately washed vegetables were implicated as the source of *E. coli* O157:H7 infection following isolation of the pathogen from a garden fertilized with manure (Cieslak *et al.*, 1993). The use of manure as fertilizer could explain foodborne outbreaks of *E. coli* O157:H7 associated with unpasteurized apple cider, potatoes, radish sprouts and lettuce (Morgan *et al.*, 1988; Besser *et al.*, 1993; Chapman *et al.*, 1997; Ackers *et al.*, 1998; Michino *et al.*, 1999). Manure may be a risk factor for transmitting *E. coli* O157:H7 to produce grown on manure-fertilized soil and may serve as a source for the maintenance of *E. coli* O157:H7 among cattle herds (Chapman *et al.*, 1997; Chart, 1998).

Given that *E. coli* O157:H7 can survive in manure and manure slurry for extended periods of time, proper manure management is of utmost importance in preventing the spread of this organism to the environment, food and animal crops, and back to cattle. Composting is an effective method for eliminating pathogens such as *E. coli* O157:H7 from manure. In preliminary studies, pathogenic organisms such as *Giardia* and *Salmonella* were eliminated from compost after 14 days, whereas *Campylobacter* and *E. coli* O157:H7 were eliminated after 7 days (Olson, 2000). Few pathogens are able to withstand the heat generated during the composting process, but it is important that 60°C be attained and maintained for threshold periods in all parts of the compost pile. Pell (1997) reported that drying manure was also effective for reducing the presence of pathogens.

2.3.3.4 *Soil*

Inoculated at a rate of 106 cfu g^{-1} of soil, *E. coli* O157:H7 survived in loam and clay soils for 25 weeks and in sandy soil for 8 weeks (Fenlon *et al.*, 2000). The organism was detectable for up to

7 days after incorporation into the uppermost 2.5 cm of the soil, and for up to 7 days on grass plots inoculated with a faecal slurry from dairy cattle at an application rate of *E. coli* O157:H7 of 660 cfu m^{-2}. Approximately 2% of the initial inoculum of *E. coli* O157:H7 was transported to the deeper layers of the soil. Transport was mainly associated with rainfall, which occurred 3 and 7 days after slurry application, and which led to a cumulative loss of 7% of the applied *E. coli* O157:H7 (Fenlon *et al*., 2000).

Maule (2000) measured the persistence of *E. coli* O157:H7 in soil using cores as a model system. Survival of the organism was greatest in soil cores containing rooted grass; viable numbers declined from 108 cfu g^{-1} soil to between 106 and 107 cfu g^{-1} soil after 130 days at 18°C.

Tilling practices, soil type and method of pathogen delivery were each found to affect vertical transport of *E. coli* O157:H7 in soil (Gagliardi and Karns, 2000). Steady rainfall was applied to intact (no-till) and disturbed (tilled) soil cores treated with manure inoculated with *E. coli* O157:H7. Manure enhanced the survival of *E. coli* O157:H7 in intact (no-till) soils but not in disturbed (tilled) soils. High levels of recovery and low leachate levels of *E. coli* O157:H7 from clay loam soils as compared to silt loam or sandy loam soils indicated that growth of *E. coli* O157:H7 in soil could occur if leaching losses were minimal. The research confirmed vertical movement of the organism through the soil for more than 2 months after initial application, provided soil pores did not become clogged. This migration represents another possible route of contamination of food and water (Gagliardi *et al*., 2000).

2.4 Occurrence of *E. coli* O157:H7 in Foods

E. coli O157:H7 has been isolated from beef, beef products, milk and milk products, and these products are implicated as sources of human infection. Besides, *E. coli* O157: H7 have also been

isolated from lamb and lamb products and is implicated as a potential vehicle of infection to humans (Chapman *et al*., 2000). A wide variety of food items, mainly vegetables or fruits contaminated indirectly with animal manure have also been identified as sources of human infection (Chapman *et al*., 1997). The Centres for Disease Control and Prevention of the United States has listed the following foods as having been found to be vehicles of *E. coli* O157:H7 infection: beef (roast, hamburger, ground beef, beef steak), venison jerky, pizza, sandwich, tacos, apple cider, milk, chicken soup, potato salad, salami, lettuce leaf, and ice/water (CDC, 2006). Cheese and yoghurt were also implicated in outbreaks of EHEC infections. Home-made hamburgers were particularly implicated as the risk for *E. coli* O157: H7 infection as they are typically prepared just before or at the same time as the rest of the meal- providing an opportunity to cross-contaminate other meal items (Mead *et al*., 1998; Russell *et al*., 2000).

2.4.1 Survival of *E. coli* O157:H7 in fermented foods

Fermentation, which results in a pH decrease due to the production of organic acids such as lactic acid, has been widely used to ensure safety and keeping quality of fermented foods. Other good attributes of fermented milk include pleasant flavours, aroma, texture and improved cooking and processing properties (Holzapfel, 2000). Several studies have demonstrated that *E. coli* O157:H7 can survive in a variety of fermented foods, with acid adapted strains, surviving even better than non-adapted strains. The implication of fermented foods in the outbreaks of foodborne diseases caused by *E. coli* O157:H7 has drawn attention of researchers to intensify their investigation on the acid resistance of this microorganism.

Acid adaptation, which is acquired after exposure of bacterial cells to mild acidic conditions, enhances survival of microorganisms in fermented food. During meat fermentation, adapted cells

showed an increased survival compared to non-adapted cells. Non adapted cell populations decreased to 120 cells/g, while adapted cell populations decreased to only 5 x 10^3 cells/g (Leyer *et al*., 1995).

Tsegaye and Ashhenafi (2005) demonstrated that *E. coli* O157:H7 can survive during processing and storage of *Ergo*, a traditional Ethiopian sour milk. In milk co-inoculated with Lactic acid bacilli, initial ($\log_{10}$ 6 cfu/ml) *E. coli* O157:H7 counts grew to $\log_{10}$ 6.5 cfu/ml after 24 hours, with levels decreasing to $\log_{10}$ 3.2 cfu/ml after 72 hours. Post fermentation inoculation of *E. coli* O157:H7 resulted in complete elimination of the initial population of $\log_{10}$ 6 cfu/ml after 36hours at ambient temperature while at refrigeration temperatures, 2.2-3.3 $\log_{10}$ cfu/ml were detectable after 72hours. Survival of *E. coli* during fermentation was attributed to development of acid resistance during fermentation while the low survival of test strain, at ambient temperature when inoculated after fermentation, was attributed to failure to develop acid resistance and to the high level of antimicrobial substances at inoculation. Lack of activity of both LAB and *E. coli* O157:H7 at refrigeration temperature was thought to be reason for the low reduction in counts under refrigeration. *E. coli* O157:H7 also survived during fermentation and storage of yoghurt at 4^0C for 7 days (Massa *et al*., 1996).

Several studies have demonstrated that low temperature enhances the survival of *E. coli* O157:H7 at low pH (Bachrouri *et al*., 2002). This is due to the fact that low pH increases the proportion of undissociated acid present and these traverse the plasma membrane into the higher pH of the cytoplasm, where they dissociate, acidifying the cytoplasm and release acid anions. Accumulation of acid anions in the cell disrupts intracellular processes (Russel, 1992). To survive low temperature, bacteria increase the proportion of unsaturated fatty acids in the cell hence preventing formation of gel-like fluids in normally fluid components (Berry and

Foegeding, 1997). Gel-like fluids hinder proper functioning of proteins and results in bacterial membrane leakage. Production of cold shock proteins is another mechanism used by bacteria to survive low pH by bacteria (Jones and Inouye, 1994). The exact function of cold shock proteins is not known but it is assumed that they assist to overcome the protein synthesis by binding RNA during transcription and facilitate initiation of translation (Jones *et al*., 1994).

In contrast, Feseru and Nyati, (1990) reported that *E. coli* survived better at 20^0C than at 5^0C in Lacto, a Zimbabwean fermented milk. *E. coli* counts could not be detected after 24 hours at 5^0C storage whereas only one or two $\log_{10}$ cfu/ml reduction in counts was observed in *E. coli* stored at 20^OC.

2.5 Modes of Transmission of *E. coli* O157:H7 to Humans

2.5.1 Contaminated foods

Contaminated and undercooked ground beef has been the principal vehicle implicated in *E. coli* O157:H7 infections (Borczyk *et al*., 1987; Bell *et al*., 1994). Faecal contamination of carcasses during slaughter and processing is the likely route by which *E. coli* O157:H7 is transferred to beef (Chapman *et al*., 1993; Elder *et al*., 2000). Grinding the beef may further compound the problem by introducing the pathogen into the interior of ground meat patties where it is more likely to survive inadequate cooking (Boyce *et al*., 1995; Mead *et al*., 1998). Ground beef often comprises meat from many carcasses, thus a few infected animals could potentially contaminate a large quantity of ground beef (Boyce *et al*., 1995).

Numerous foods other than ground beef have been linked to *E. coli* O157:H7 outbreaks. Cross-contamination as a result of contact with bovine products or contamination with faeces of wild or domestic animals has been suspected in the majority of these outbreaks (Armstrong *et al*., 1996; Mead *et al*., 1998).

The levels of contamination in the food samples incriminated in *E. coli* O157:H7 outbreaks support the hypothesis of a low infectious dose (Armstrong *et al*., 1996; Neill, 1997). A community outbreak associated with beef burgers in Wales in 1993 was used to estimate the presence of *E. coli* O157:H7 in the burgers. Fewer than two organisms per 25 g sample of meat caused illness, implying that the infectious dose of *E. coli* O157:H7 could be as low as 10 organisms (Willshaw *et al*., 1994). The infectious dose of *E. coli* O157:H7 is also affected by variables such as stomach pH, food composition and host susceptibility (Cassin *et al*., 1998; Gannon, 1999).

In 1994, 12.5% of outbreaks were linked to food sources other than beef products; in 1996 the proportion increased to 21%. The proportion of outbreaks linked to beef products has decreased, from 28% in 1994 to 14% in 1996. The acidic foods confirmed as sources of outbreaks include unpasteurized apple juice and apple cider, mayonnaise and yogurt (Feng, 1995). Fresh-pressed, unpasteurized, unpreserved apple cider was first identified as a vehicle for *E. coli* O157:H7 in an outbreak in Massachusetts in 1991, although HUS was first linked to apple juice in 1982 (Besser *et al*., 1993). In October 1996, two separate outbreaks associated with drinking unpasteurized apple cider occurred, one in Connecticut and the other in the western U.S. The Connecticut outbreak involved 14 cases and was associated with drinking a specific brand of cider (Mshar *et al*., 1996). The second outbreak involved 66 persons in multiple states in the western U.S. and

was associated with drinking a specific brand of unpasteurized apple juice or the brand's juice mixtures containing apple juice (CDC, 1999).

Raw milk can be a vehicle of transmission for *E. coli* O157:H7, but confirmed outbreaks have been few. The presumed mechanism of contamination is faecal contamination during milking. Two outbreaks associated with raw milk have been documented by the CDC, one in 1992 with 9 cases and the other in 1993 with 6 cases. Both outbreaks occurred in Oregon and were traced to two specific dairies which were licensed to sell raw milk (USDA, 1994).

Milk from dairy cows, sheep, and goats may be contaminated with *E. coli* and other bacteria from the environment. Proper pasteurization will kill these bacteria. Outbreaks of *E. coli* O157:H7 due to contaminated dairy products are usually associated with unpasteurized milk but there have been some cases of post-pasteurization contamination (Doyle *et al*., 2006)

Manure is a valuable fertilizer for crops but manure containing *E. coli* O157:H7 may be a source of contamination for vegetables or fruits that are not normally cooked before eating. Field and greenhouse experiments have demonstrated that both *E. coli* O157:H7-contaminated manure and irrigation water may cause contamination of vegetables. Onions and carrots grown in soils treated with contaminated manure or irrigated with contaminated water had detectable levels of *E. coli* O157:H7 on their subterranean parts for 2.5 to 5.5 months (Islam *et al*., 2005). Lettuce grown in soil amended with contaminated manure did not contain *E. coli* O157:H7 in leaves (Johannessen *et al*., 2004; Johannessen *et al*., 2005) but spray irrigation of lettuce with contaminated water deposited *E. coli* O157:H7 on lettuce leaves and these bacteria persisted for up to 30 days (Solomon *et al*., 2003). Experiments with shredded lettuce, carrots, and cucumbers

demonstrated that *E. coli* O157:H7 could survive and grow on these vegetables even under modified atmospheres used in commercial packaging (Abdul-Raouf *et al*., 1993).

2.5.2 Contaminated water

Outbreaks of *E. coli* O157:H7 infections have been associated with drinking water and with swimming (Swerdlow *et al*., 1992; Keene *et al*., 1994; Ackman *et al*., 1997; Paunio *et al*., 1999). In 1989, a large outbreak of *E. coli* O157:H7 infection occurred in Cabool, Missouri. This outbreak was associated with drinking water from an unchlorinated water supply (Swerdlow *et al*., 1992). The outbreak resulted in 243 cases of illness and four deaths. Epidemiological studies of three swimming-associated outbreaks implicated fresh-water lakes as the source of *E. coli* O157:H7 infection, with illness presumably due to bathers swallowing small amounts of contaminated lake water (Swerdlow *et al*., 1992; Keene *et al*., 1994; Paunio *et al*., 1999). An outbreak of *E. coli* O157:H7 infection in June, 1998 was associated with visiting a water park near Atlanta, Georgia, and affected residents of eight states. Evidence suggested that contamination of park water by human faeces was the source of infection (Georgia Department of Public Health, 2002). In May 2000 the system supplying the drinking water to the town of Walkerton, Ontario, became contaminated, primarily with *E. coli* O157:H7 and *Campylobacter jejuni*, resulting in more than 2300 illnesses and seven deaths (The Walkerton Inquiry, 2002).

2.5.3 Person -to- person transmission

It has been suggested that the spread of *E. coli* within families and in institutionalized settings may occur through means other than foodborne transmission (Doyle, 1991; MacDonald *et al*., 1996). Approximately 11% of the illnesses recorded during a large *E. coli* O157:H7 outbreak in the United States in 1993 due to contaminated beef burgers were determined to be secondary

infections (Bell *et al*., 1994). In Wales, between 1990 and 1998, 17% of the identified cases of *E. coli* O157:H7 infections were the result of contact with an index case (Parry and Palmer, 2000). The household transmission rate of *E. coli* O157:H7 infection in Wales between 1994 and 1996 was estimated to be 7%, with children aged 1–4 yr and adults aged 15–34 yr most likely to contract infection from an index case through household contacts. This pattern of transmission suggests that person -to-person spread is likely via faecal-oral routes, as opposed to foodborne transmission.

Person-to-person spread of *E. coli* O157:H7 has also been identified as the primary means of transmission of infection in day care centres (Swerdlow *et al*., 1997). Person-to person transmission has been identified in secondary cases of illness in a nursing home outbreak and has been implicated in an outbreak involving venison jerky (Ryan *et al*., 1986; Keene *et al*., 1997)

2.5.4 Bovine –to- human transmission

Three incidents of apparent direct transmission of *E. coli* O157:H7 from bovines to humans have been reported (Renwick *et al*., 1993; Synge *et al*., 1993; Rice *et al*., 1996). In Ontario, Canada, in 1992, a 13 month old boy was hospitalized with haemorrhagic colitis following prolonged contact with calves on a dairy farm. In Scotland in 1993, a 15 month old child who lived adjacent to a farm became ill with *E. coli* O157:H7 (Synge *et al*., 1993). It was suggested that the family dogs may have carried the organism into the household, resulting in the infection of the child. An additional case of *E. coli* O157:H7 infection acquired from livestock occurred in a 10 year old boy who was actively involved in raising livestock (Rice *et al*., 1996).

In 1999, an outbreak of *E. coli* O157:H7 infection among visitors to a petting zoo in Ontario resulted in 159 illnesses and four confirmed deaths (Warshawsky *et al*., 1999). Direct contact

with farm animals as a result of school visits to farms resulted in outbreaks of *E. coli* O157:H7 infection in Pennsylvania and Washington in the spring and fall of 2000 (CDC 2001). Children were allowed to touch cattle, calves, sheep, goats, llamas, chickens and pigs. Lack of appropriate hand washing prior to eating and eating while petting the animals may have been associated with infection (Warshawsky *et al*., 1999; CDC, 2001).

2.6 Risk Factors for Human Cases of *E. coli* O157:H7

E. coli O157:H7 infections occur in all age groups, with the highest incidence rate in children less than 5 years old. In 1987, the first year that Washington State required reporting of *E. coli* O157:H7 infection, the highest age-specific incidence rate was among children younger than 5 years (6.1 cases per 100,000 population per year) and lowest for adults 50-59 years of age (0.5 cases per 100,000) (Ostroff *et al*., 1989). Higher incidence of infection in young children may in some part be due to the greater likelihood of their being brought to medical attention. Variation of incidence rates by age may also be related to variation of exposure to the agent by age. The demographic profile of people exposed to a specific vehicle (such as swimming water) affects the demographic profile of outbreak cases. The young and the elderly are more often affected by the serious complications of *E. coli* O157:H7 infection, HUS and TTP. Consequently, the young and the elderly have the highest morbidity and mortality rates from *E. coli* O157:H7 infection (Su *et al*., 1995).

The most commonly identified risk factor in case-control studies of sporadic *E. coli* O157:H7 illness was consumption of undercooked ground beef. Other risk factors identified were consumption of ground beef in a non-commercial setting such as a picnic or “special event”, drinking of well water, swimming, handling animal faeces, close contact with a person with

diarrhoea, and failure to wash one's hands after handling raw ground beef (Armstrong *et al.*, 1996). In 1992-1993 the Food and Drug Administration sponsored a national telephone survey of 1,620 respondents at least 18 years of age, to assess the prevalence of selected self-reported food consumption and preparation behaviours associated with increased risks of food-borne illness. Consumption of undercooked hamburger was reported by 23% of respondents, and 25% of respondents reported that after cutting raw meat or chicken, they use the cutting board again without cleaning it. These results indicate that unsafe food preparation and consumption behaviours are common in the USA (Klontz *et al*., 1995).

Reymond *et al*. (1996) used assays to detect antibodies to *E. coli* O157 lipopolysaccharide (LPS) and verotoxin 1 (VT1) to determine and compare exposure of dairy farm residents in southern Ontario, Canada and in urban residents of Toronto, Canada. The frequency of O157 LPS antibodies was significantly higher in dairy farm residents (12.5%) than in urban residents (4.7%). The difference between the groups was even greater for VT1 neutralizing antibodies, with detection in 42% of dairy farm residents and only 7.7% in urban residents. These findings indicate that dairy farm residents are at higher risk for *E. coli* O157 VT+ exposure.

A case-control study was conducted in an Inuit community in northern Canada to evaluate risk factors for childhood HUS and gastroenteritis during an epidemic of *E. coli* O157:H7 infection in 1991. Results of the study indicated that in the 7 days before the onset of gastrointestinal symptoms, children with HUS and those with uncomplicated gastroenteritis were 9 times more likely to have been exposed to a family member with diarrhoea than were the healthy control subjects (Rowe *et al*., 1994). This study illustrates the importance of person-to-person transmission.

2.7 Clinical Manifestations of *E. coli* O157:H7 Infections

Infection with *E. coli* O157:H7 causes three principal manifestations of illness in humans: haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (Doyle *et al*., 1989; Griffin *et al*., 1991; Su *et al*., 1995; CDC, 2006; WHO, 2005).

2.7.1 Haemorrhagic colitis (HC)

Haemorrhagic colitis (HC) is the principal illness caused by *E. coli* O157: H7, diarrhoea being the most common clinical presentation, with an incubation period of 1-14 days, which is frequently, but not invariably, bloody in nature, commonly accompanied by severe, cramping abdominal pain. Vomiting occurs in about half of patients, but fever is uncommon, and the illness resolves in 5-10 days (Eslava *et al*., 2003; WHO, 2005). In some cases, the diarrhoea symptoms may be very severe, consisting of all blood and occurring in every 15-30 minutes. This severe haemorrhagic colitis (HC) may be seen in the very young or old patients (Coia, 1998). However, some infections may also be completely asymptomatic (Eslava *et al*., 2003). The disease is distinguished from dysentery described in shigellosis or invasive *E. coli* gastro-enteritis by the lack of fever and the bloody discharge resembling lower gastrointestinal bleeding (Padhye *et al*., 1992).

2.7.2 Haemolytic uremic syndrome (HUS)

Haemolytic uremic syndrome (HUS) is the most common cause of acute renal failure in children less than 4 years of age and a proportion of affected children die of this illness (Mead *et al*.,

1998). Padhye *et al*. (1992) stated that the disease is a tirade of an acquired haemolytic anaemia, thrombocytopenia and renal failure that occurs acutely in otherwise healthy individuals. In the 1980s, this proved to be correct when a novel category of diarrhoeagenic *E. coli*, so called enterohaemorrhagic *E. coli* (EHEC), that was causally associated with the syndrome of hemorrhagic colitis and subsequently with HUS was discovered (Karmali, 1989). Padhye *et al*. (1992), reported that the pathogenesis of HUS appears to be associated with toxin which damages endothelial cells, thus triggering the clotting mechanism.

Progression to post-diarrhoeal HUS occurs in a small percentage of *E. coli* O157:H7 cases. Estimates of the rate of HUS development vary, but it is probably 2–7% among sporadic cases and possibly as high as 20% in outbreaks (Besser *et al*., 1993,Boyce *et al*., 1995; Griffin *et al*., 1991; Mead *et al*., 1998). Around 90% of HUS cases are associated with diarrhoea prior to development of HUS (Mahon *et al*., 1997). HUS has three classic components: microangiopathic haemolytic anaemia, thrombocytopenia, and acute renal failure (Besser *et al*., 1999; Karmali *et al*., 1985; Mead *et al*., 1998). The annual incidence of HUS in the U.S. and in Canada is approximately three cases per 100,000 in children less than five years old (Mahon *et al*., 1997; Rowe *et al*., 1991).

Other complications of HUS include acute neurological complications: stroke, seizure, lethargy, hemiparesis, decerebrate posturing, and coma (Besser *et al*., 1999; Mead *et al*., 1998; Pickering *et al*., 1994). Pancreatitis, diabetes mellitus, pleural effusions, and pericardial effusions occur as rare complications. The long-term sequelae of HUS include cholelithiasis, colonic stricture, chronic pancreatitis, glucose intolerance, and cognitive impairment (Mead *et al*., 1998). Multiple systems can be affected and the range of symptoms and complications can be non-specific, complicating quick diagnosis.

2.7.3 Thrombotic thrombocytopenic purpura (TTP)

Thrombotic thrombocytopenic purpura is similar to HUS except the central nervous system (CNS) is involved (Padhye *et al*., 1992); it is a further complication of HUS. It is a syndrome usually occurring in adults that consists of microangiopathic haemolytic anaemia, profound thrombocytopenia, fluctuating neuralgic signs, fever, and mild azotemia (Boyce *et al*., 1995; Griffin *et al*., 1991; Mead *et al*., 1998).

TTP is a diagnosis sometimes given to patients with STEC O157:H7 infection. TTP includes most of the clinical findings found in HUS with the exception of prodromal diarrhoea and less pronounced renal impairment. Neurological findings are more pronounced in TTP case patients. Diagnosis of TTP occurs more frequently in adults and is probably the same syndrome as HUS when caused by STEC O157:H7 (Besser *et al*., 1999).

Patients often develop blood clots in the brain and death frequently results. Other unusual clinical complications associated with *E. coli* O157:H7 infection include haemorrhagic cystitis and banalities, convulsions, sepsis with another organism, and anaemia (Rowe *et al*., 1991).

2.8 Pathogenesis of *E. coli* O157:H7

The pathogenesis of *E. coli* O157:H7 infection is believed to be associated with the formation of toxins. These toxins are called verotoxins because of their toxicity to vero cells. All of the strains of *E. coli* O157:H7 produce one or two verotoxins (VT1 and VT2) (Johnson *et al*., 1983 and Abdul-Raouf *et al*., 1994). Verotoxin 1, which is also called Shiga-like Toxin I, is immunologically indistinguishable by ouchterlong immunodiffusion analysis from the toxin produced by *Shigella* dysentery type 1 (Shiga toxin) and possesses the same subunit structure as shiga toxin. VT1 is composed of two subunits, subunits A and B, which have molecular weights

of 29.000 to 31.000 and 5.000 to 6.000, respectively, and has an isoelectric point of 7.03 (O´Brien and Leveck, 1983).

Although the mechanism of pathogenicity associated with HC, HUS, and TTP caused by *E. coli* O157:H7 is not yet fully understood, there are important factors thought to influence the virulence of the EHEC serotypes. One is the intestinal attachment and effacing adherence mechanism, encoded by the eae A gene, which is homologous to the eaeA gene of EPEC (Knutton *et al*., 1989). The product of this gene, a 97-kDa protein known as intimin, is necessary for the production of attaching and effacing (AE) lesions, characterized by localized adherence to and destruction of microvilli, rearrangement of cytoskeletal elements, and "pedestal" formation, in which filamentous actin accumulates at the site of intimate bacterial attachment to the intestinal wall (Donnenberg and Kaper, 1992). Formation of these AE lesions allows a subset of *E. coli* O157:H7 organisms to further invade the epithelial cells, resulting in bowel inflammation, perforation, and necrosis, manifested as bloody diarrhoea (Griffin, 1991).

The other key virulence factor is the production of one or more verotoxins (VT) that are cytotoxic to tissue cells (Bettelheim, 1996). Konowalchuck *et al*. (1977) reported that culture supernatants from some strains of *E. coli* were toxic to Vero cells obtained from an African green monkey kidney cells. O'brien *et al*. (1982) reported that one *E. coli* O157:H7 VT was immunologically indistinguishable from Shiga toxin. Biological activities, such as mouse lethality and enterotoxicity of the two toxins, also were virtually identical. Thus, the term Shigalike toxin (SLT) was coined to describe the toxin. O'brien *et al*. (1983) and Johnson *et al*. (1983) found that *E. coli* O157:H7 produced high levels of this verotoxin (VT).

Another *E. coli* O157:H7 VT was identified by Strockbine *et al.* (1986). This toxin was not neutralized by antisera against Shiga toxin. To differentiate the two, the first toxin, indistinguishable from Shiga toxin, was deemed SLT-1 or VT-1, while the other toxin was referred to as SLT-2 or VT-2. A third cytotoxin was described by Padhye *et al.* (1992), which also was not neutralized by Shiga antisera. Other variants of the *E. coli* VT have been identified, as well (Marques *et al*., 1987; Oku *et al.*, 1989). A study by Ogasawara *et al*. (1987) determined that the pathogenic mechanism of both VT-1 and Shiga toxin was the blockage of protein synthesis by inhibiting elongation factor-1 (EF-1)-dependent aminoacyl binding of t-RNA to 60S ribosomal subunits. Additional studies determined that VT-2 acted in the same manner (Ogasawara *et al*., 1987). Data collected from human cases of *E. coli* O157:H7 infection suggest that VT-2 is a more important virulence factor than VT-1 for progression of the infection into HUS (Thomas *et al*., 1993; Scotland *et al*., 1987).

2.9 Detection and Isolation

Levine (1987), reported that *E. coli* O157:H7 is different from other *E. coli* not only from certain clinical and epidemiological stand points but also in some bacteriological features, including ß-glucuronidase activity, sorbitol fermentation, raffinose, dulcitol reactions and production of enterohaemolysin. Most procedures used traditionally to detect faecal coliforms and subsequently *E. coli* use incubation temperatures in the range from 44 to 45 °C. However, *E. coli* O157:H7 grows poorly at this particular temperature range (Doyle and Schoeni, 1984).

The isolation of *E. coli* O157:H7 is important to both clinical and food microbiologists. Several methods have been developed for detection and isolation of *E. coli* O157:H7 in foods and in clinical samples.

Selective reagents can prevent or inhibit the recovery and subsequent multiplication of injured cells and direct inoculation, either into selective enrichment broths or onto selective agar plates is still used in many methods for *E. coli* O157 detection and enumeration. Six rapid methods for the detection of *E. coli* O157 in food (BAX *E. coli* O157, Reveal 8 *E. Coli* O157 screening test, VIP EHEC, VIDAS *E. coli* O157 (ECO), EHEC-Tek and Tecra *E. Coli* O157 Visual Immunoassay), were evaluated using beef burgers, parsley and fermented meat artificially contaminated with injured cells.

Methods using direct selective enrichment, with or without an elevated incubation temperature gave false-negative results. The incorporation of a non-selective pre-enrichment medium improved the detection rates of these assays by up to ten fold (Blackburn and McCarthy, 2000).

A unique characteristic of *E. coli* O157:H7 is its inability to ferment sorbitol within 24 h. Of incubation, through 80.3 % of *E. coli* strains are sorbitol fermentors (Wells *et al*., 1983; Ewing 1989; Krishnan *et al*., 1987 and Ratnam *et al*., 1988). This characteristic has been used to differentiate *E. coli* O157:H7 from other *E. coli* strains (Wells *et al*., 1983; Farmer and Davis, 1985; March and Ratnam, 1986; Krishnan *et al*., 1987; Kleanthous *et al*., 1988).

Okrend *et al*. (1990) found that 25 % of all sorbitol negative colonies picked, many of which were *E. coli,* were *ß*-glucuronidase positive. Since approximately 97 % of all *E. coli* are *ß* glucuronidase positive (Kilian and Bulow, 1979), but *E. coli* O157:H7 is *ß*-glucuronidase negative (Doyle and Schoeni, 1984; Krishnan *et al*. 1987; Ratnam *et al*., 1988). Several researchers have reported on the specificity of *ß*-glucuronidase as a differential test for *E. coli* (Feng and Hartman, 1982; Robison, 1984; Petzel and Hartman, 1989; Moberg, 1985). Furthermore some research have incorporated the compound 4-methylumbelliferyl *ß*-D-glucuronide (MUG) as an indicator into coliform detection and/or enumeration media to achieve

simultaneous detection of both coliforms and *E. coli* (Dahlen and Linde, 1973; Feng and Hartman, 1982; Trepeta and Edberg, 1984; Robison, 1984; Petzel and Hartman 1989; Moberg, 1985; Freier and Hartman 1987; de Boer, 1998; and Reinders *et al*., 2000).

However, Doyle and Schoeni (1984), found that *E. coli* O157:H7 does not hydrolyse the MUG indicator, hence, the organism would not be detected by this method. Phenotypic characterisation of *E. coli* O157:H7 illustrated by lack of sorbitol fermentation within 24 hours. and MacConkey sorbitol agar media which is used as deferential media for *E. coli* O157:H7 (March and Ratnam, 1986; Kleanthous *et al*., 1988).

The supplementary biochemical markers such as ornthine and lysine in conjugation with sorbitol improve the specificity of the basic sorbitol screening procedure from 11.3 to 33.6 % by excluding the organisms with negative ornithine and lysine decarboxylase reactions

A rapid latex agglutination assay (*E. coli* O157:H7 latex, Oxoid, Ltd., Hampshire, England), has been developed for the rapid presumptive detection of *E. coli* O157:H7 (Padhye and Doyle, 1992).

Abdul-Raouf *et al*. (1996) isolated *E. coli* O157:H7 from ground beef, chicken, lamb, milk samples. He examined *E. coli* O157:H7 isolate for sorbitol and ß-glucuronidase and subjected the purified colonies of *E. coli* O157:H7 to biochemical and serological confirmation tests which includied API20E miniaturised diagnostic kit (Analy Tab, Div, of Sherwood Medical, plain view, NY), the *E. coli* O157:H7 latex agglutination assay (Unipath- Oxid, Ogdenberg, NY) and the Bacto *E. coli* H antiserum H7 assay (Difco).

Recently, Ali *et al*. (1999) developed a new medium for differentiation between *E. coli* O157:H7 and other bacteria (*E. coli* O157:H7 medium: Salah/Fung EOH medium). Metanil yellow (0.001 g/l) and aniline blue (0.002g/l) were found as best combination for differentiation between *E.*

coli O157:H7 and other selected bacteria when added to the basal SMAC medium (excluding natural red and crystal violet). On the Salah/Fung EOH medium, *E. coli* O157:H7 produced a yellow colour colony with a yellow zone whereas *E. coli* produced a blue colour and other bacteria produced green to blue colour colonies (Bader, 2000).

For epidemiological purpose/surveillance O157 VTEC can be distinguished by phage typing (Ahmed *et al*., 1987) which can be combined with typing of the VT genes to give added discrimination. Other methods such as plasmid profile analysis, pulsed field gel electrophoresis and multilocus enzyme electrophoresis may further differentiate O157 VTEC and can be applied to VTEC of other serogroups. Restriction fragment length polymorphism analysis of genomic DNA probed with phage ë or the DNA of a VT encoding phage have also been used to differentiate O157 VTEC strains (Fernandez, 2008).

2.10 Prevention and Control

As bovine reservoir is believed to be of major importance to the occurrence of *E. coli* O157:H7 infections in humans, reduction of this organism in cattle is the most effective means to control its spread through meat, milk or other transmission routes. Control has been challenged in the food industry, particularly beef industry, by the very low infective dose and ability of the organism to withstand low pH. To ensure the effective implementation of the control measures, they may be categorized as short-, middle-, and long term priorities (Reinders *et al*., 2001). Short-term priorities will deal with the matters of immediate concern like the outbreak situations. Mid-term priorities will include surveillance of the organism in the farm, the animals and the food. Likewise, long-term measures will include the integration and application of the finding of

the above surveillance for the formulation of effective control measures like implementation of hygienic farm management and feeding practices.

It is generally felt that control strategies for this and other foodborne pathogens must extend along the food-production continuum (from 'farm to fork') and include not only slaughter and processing steps but also measures to control the pathogen in cattle populations. At the other end of this continuum, steps can be taken to limit the growth and survival of *E. coli* O157:H7 during transport, storage and retail display and to provide effective decontamination and cooking procedures.

2.10.1 On farm control

Hygienic post-slaughter processing practices effectively reduce carcass contamination with *E. coli* O157: H7, but on farm (pre-slaughter) intervention strategies offer the opportunities to reduce the microbial load in animals before they enter the food chain. Bovine faeces are a potential source for spreading *E. coli* O157: H7 to human food chain as well as to the environment. Contamination of the environment and food by *E. coli* O157:H7 can be prevented through effective control of carriage of this organism in cattle and proper handling or usage of the manure from these animals (Wang *et al*., 1996). It has been suggested that pre-slaughter reduction of *E. coli* O157: H7 prevalence in cattle would result in considerable reduction of contamination of beef and consequent human disease (Jordan *et al*., 1999).

2.10.1.1 *Feed and water hygiene*

Cattle and other farm animals are exposed to *E. coli* and other faecal organisms, most commonly via feed and water that might have been contaminated with these organisms. A wide variation in the level of contamination of feed and water has been found suggesting this level is potentially

subjected to management practices of the farm (Hancock *et al*., 2001). Feed might have been contaminated before purchase or during on-farm storage or mixing. Purchased feed are also implicated as primary vehicle for regional dissemination of *E. coli* O157: H7. One of the reasons for increased faecal shedding of *E. coli* O157: H7 from animals during summer months may be increased *E. coli* counts in trough water, and growth of the organism supported by wet grain mixes, during this time period (Hancock *et al*., 2001). Theoretically, it appears that implementation of strict feed hygiene practice is the solution, but is difficult on practical grounds. Water-borne infections may be avoided by frequent cleaning of the water troughs to prevent the persistence of *E. coli* O157 in water (Conedera *et al*., 2001).

2.10.1.2 *Reducing faecal shedding*

Competitive inhibition: Probiotics are the bacteria that are administered orally for the competitive inhibition of target organism. It has been practiced widely in monogastric animals, mainly in poultry production. Limited information is available regarding the ruminants. It has been suggested that administration of bovine commensal *E. coli* that colonize that same anatomic locations as *E. coli* O157: H7 and produce toxic metabolites like colicin may reduce the carriage of *E. coli* O157:H7 in the bovine digestive tract (Harmon *et al*., 1999). Zhao *et al*. (1998) demonstrated that inoculation of cattle with non-toxigenic *E. coli* and *Proteus mirabilis* can reduce the level of carriage and faecal shedding of *E. coli* O157: H7 in most animals. Another advantage of administering probiotics in ruminants appears to be their competition with *E. coli* O157: H7 in other locations within the farm environment, such as in the feedlot manure (Harmon *et al*., 1999). These findings are very promising since even if such probiotic preparation reduces only 50% of the faecal shedding of the *E. coli* O157: H7 that would have a marked influence on

ecology of this organism which will in turn reduce the level of environmental exposure to other animals (Hancock *et al*., 2001).

Another way of inhibiting the survival of *E. coli* in rumen is by creating an unfavourable environment, e.g. through elevating the volatile fatty acids level, either by dietary supplementation of VFAs or stimulating the VFA production from commensal anaerobes (Duncan *et al*., 1999). It has been suggested that ruminal and intestinal VFA concentrations limit the proliferation of *E. coli* (Callaway *et al.*, 2003).

Pre-slaughter feeding management: Cattle feeding practices may be manipulated to decrease the gut proliferation and faecal shedding of *E. coli*. The extent of carcass contamination is believed to be dependent on the number of *E. coli* O157:H7 shed per gram of faeces at slaughter (Gannon, 1999). As *E. coli* shedding in faeces is dependent on the ruminal count of the organism, manipulating its survival in the rumen before transport for slaughter appears to be an effective way of reducing the counts of *E. coli* in the faeces (Gregory *et al*., 2000). Faecal shedding is believed to be facilitated by survival of the passage of the organism through the acidic abomasum and subsequent localization in the large intestine (Brown *et al*., 1997). When cattle are fed with high grain-based rations, some starch reaches the hindgut escaping the ruminal digestion, which is believed to facilitate the multiplication of *E. coli* there (Callaway *et al*., 2003). This also increases the acid-resistance of the organism which will ultimately facilitate its survival on the low pH environment of human stomach (Russell *et al*., 2000). It has been widely accepted that abrupt switching of cattle from a high grain-ration to high-quality hay-based diet reduces the generic *E. coli* and *E. coli* O157: H7 population, also their acid tolerance (Russell *et al*., 2000, Hancock *et al*., 2001; Callaway *et al*., 2003). This method is believed to be having

widespread preslaughter application in the cattle industry, also because the effect is almost immediate (Russell *et al*., 2000).

Deprivation of cattle from feeding results in proliferation of coliforms in the bovine rumen and subsequent increase in faecal shedding (Harmon *et al*., 1999; Brown *et al*., 1997; Gregory *et al*., 2000). As feed withdrawal or fasting results in lower volatile fatty acids concentration in the rumen and hindgut (Callaway *et al*., 2003), there will no longer be the inhibitory effect of VFA on proliferation of *E. coli*. Also, long distance transport of cattle for up to 48 hours, before slaughter without food, has been shown to increase *E. coli* populations throughout the intestinal tract (Gregory *et al*., 2000). Feeding of hay or other roughages 48 hours before dispatch for slaughter may be helpful in decreasing bacterial proliferation (Gregory *et al*., 2000). This practice was also found to keep the soiling of hides to a minimum (Gregory *et al*., 2000).

Feeding of limestone to cattle may also reduce the incidence of acid tolerance in *E. coli* (Russell *et al*., 2000).

It has been suggested that if ruminal fermentation of grains can be enhanced in such a way that minimal unfermented starch reaches the colon, proliferation of *E. coli* may be prevented there. Russell *et al*. (2000) mentioned that it may be achieved by processing the grains, e.g. by heating, so that protein coating of the starch granules breaks-down and the starch becomes readily available for ruminal fermentation.

Vaccination: Several groups are working on *E. coli* O157: H7 vaccines for cattle, but none has been successful for a breakthrough. As with competitive inhibition, even a slight reduction in faecal shedding of the organism would have substantial impact on the dynamics of *E. coli* O157: H7 in a farm ecosystem (Hancock *et al*., 2001). Jordan *et al*. (1999) in a simulation study

suggested that vaccination has the greatest potential impact to reduce the shedding of *E. coli* O157 in faeces.

2.10.1.3 *Reductions in hide soiling*

As hide is implicated to be an important source of bacterial contamination of carcasses, including *E. coli* O157 (Gannon, 1999), efforts to reduce the faecal soiling of body coat seems to be important for control of this organism. Taking this fact into consideration, it has been reported that some processing plants in the USA have started to discriminate between clean and heavily soiled animals (Hancock *et al*., 1998). However, Jordan *et al*. (1999) suggested that reduction of visible hide soiling would have very small impact on reducing the *E. coli* O157 contamination of carcasses. Likewise, Byrne *et al*. (2000) also documented that pre-slaughter hide washing for 3 minutes did not significantly reduce the subsequent contamination of carcass.

2.10.1.4 *Manure recycling practices*

Bovine faeces or contaminated pastureland supports the survival of *E. coli* O157: H7 for extended periods, up to several months (Bolton *et al*., 1999). Contamination of pastureland with *E. coli* O157: H7 may be the result of farm waste recycling practices like the spreading of manure onto it or irrigating the pasture with cow manure slurry. This in turn may result in increased carriage of the organism in bovine herds (Wang *et al*., 1996), posing great risks to the final consumer of resulting milk and meat products (Bolton *et al*., 1999). So, proper management and/or treatment of manure and slurry may also be important in preventing contamination of farm environment and infection in cattle.

2.10.1.5 *Surveillance of the organism*

These measures include surveillance of the organism in animal population on farm, after transportation (i.e. shedding of VTEC, and the spread of the organism between herds or among animals entering slaughter facilities) and population dynamics in dairy farm (i.e. duration of shedding by individual animals, and the extent of on-farm VTEC transmission among animals) as well as studies of farm environment. Identification of *E. coli* O157: H7 positive cattle before slaughter is a crucial step in any on-farm control measures to reduce the risk of carcass contamination with this pathogen. In one Canadian report (Anon, 2000), it was recommended that slaughtering of cattle shedding more than 104 CFU/g of faeces of *E. coli* O157: H7, should be done separately.

2.10.1.6 *Quarantine and farm hygiene*

If a farm is suspected of harbouring high-risk infective materials, the infection should be restricted and further spread of the infection from the farm should be prevented. It may be achieved by imposing strict quarantine measures. The imposition of quarantine involves the restriction of movement of animals and animal products (with the exception of milk) as long as a farm is considered positive. Milk from such farms should be pasteurized before removal. Manure should be stockpiled, and if necessary, treated. Before spreading on land, it should be made sure by bacteriological analysis that the manure is free from the organism.

Contaminated materials within the farm should be restricted in a system which prevents proliferation of the organism and prevent animal-to-animal transmission. This can be achieved by a range of hygienic measures, including separate rearing of animals of different species (Eriksson *et al*., 2003) or age groups (Reinders *et al*., 2001), regular cleaning and disinfection of troughs and bowls, quick removal of feed leftovers and strict personal hygiene among farm

workers. All animals on the farm should be sampled every 2 weeks, as regular and repeated bacteriological examination is necessary to accurately determine that each animal is truly free from *E. coli* O157, rather than merely irregularly shedding the pathogen. In general, a farm may be considered free of *E. coli* O157 when all samples are negative for the organism over two successive samplings. Animals that are shedding the organism for more than 6 weeks or over three successive samplings are considered to be long-term shedders, and should be removed (Reinders *et al.*, 2001). However, implementations of hygienic on-farm control measures are difficult, particularly in the types of farming where different species and age groups of animals co-exist in contagious pens (Conedera *et al*., 2001).

2.10.1.7 *Eradication of the organism*

The level of infection in a herd with the strains of VTEC O157 is very dynamic. Some strains are present for long periods, while others are less persistent. Hence a farm may soon acquire new strains of the organism after completion of an eradication program. Regardless of the potentials of reinfection, eradication programmes should be carried out after a herd has been exposed to an outbreak (Reinders *et al.*, 2001). However, eradication seems to be unsuccessful for such an ubiquitous organism that is not host specific (Hancock *et al*., 2001).

2.10.2 Control at slaughter

It has been suggested that *E. coli* contaminates beef carcass in the processing plants either directly from spillage of ingesta and faecal materials onto the carcass during processing or through faecal material that is transferred from the hide or by contact with workers or equipments that have been contaminated. As *E. coli* O157: H7 has been reported in faeces, rumen contents and on the hide of cattle at slaughter (Brown *et al*., 1997; Chapman *et al*., 1997;

Wang *et al*., 1996), prevention of possible contamination of carcass is the most significant challenge to the meat processing plants. This is largely dependent on the *E. coli* O157: H7 status of the preslaughter animal, and the processing techniques which distribute the organism within or between carcasses during dressing operations. Although it is not possible to completely avoid carcass contamination during slaughter and processing, it can be minimized to a very low level by strict hygienic measures like the hazard analysis critical control points (HACCP) (Gannon, 1999).

2.10.2.1 *Controlling sources of carcass contamination*

The most important sources of contamination of carcasses following slaughter are associated with skinning and dung tying (McEvoy *et al*., 2003), and include: faeces, hide, soils, aerosols and sprays, contact with workers' hands, gloves and other equipment and accidental spillage of body fluids during evisceration. Contamination of carcass can take a number of forms (Gannon, 1999).

1. Accidental, random, visible contamination of carcasses, e.g. when faeces or intestinal contents contaminate a carcass following a knife puncture.
2. Systematic visible contamination, e.g. defined areas or zones of the carcass shown to have higher visible demerit scores than others.
3. Accidental or random invisible contamination from aerosols, dusts and sprays.
4. Systematic invisible contamination from contact, smears, aerosols, dusts and sprays.

It has been reported that most of the microbial contamination of the carcass in the processing line occurs during skinning of the hindquarters (Gill *et al*., 1998). As there is likelihood of these

organisms coming directly from faeces in the anal region, rump region is the most vulnerable for contamination with *E. coli* (Gill *et al*., 1998). However, this can be minimized by enclosing the anus and adjacent portion of the rectum or 'bung' in a plastic bag and binding this to the outer wall of the rectum (Gannon, 1999; Russell *et al*., 2000). Bell (1994) mentioned that the neck, the brisket, legs and hooves are also the areas that are highly prone to contamination with *E. coli*. This is presumably related to contact between these areas of carcass and the outside surface of the hide during skinning (Gannon, 1999; McEvoy *et al*., 2003).

Preslaughter washing: Preslaughter washing of the animals may be an appropriate technique of decontaminating animal hides, and preventing the subsequent carcass contamination. Byrne *et al*. (2000) reported that preslaughter washing of bovines for 3 minutes significantly reduced hide faecal contamination with *E. coli* O157: H7, however, it did not significantly result in lower carcass contamination.

2.10.2.2 *Cleaning contaminated carcasses*

If the contamination area is less than 2.5 cm in its greatest dimension, it should b removed either by knife trimming or by steam vacuum, and if larger than 2.5, should be trimmed away with a knife (Gannon, 1999). Visible contamination may also be reduced by washing the carcass simply with cold or warm water. Though not an effective method to eliminate all the organisms, cold water wash may decrease the microbial contamination following skinning by reducing the ability of the organism to adhere to the carcass surface. Hot water treatment is, however believed to be a more promising method to decrease the risk of *E. coli* O157: H7 contamination of beef carcasses which consists of a combined whole-carcass treatment. However, these procedures alone are not reliable enough to ensure elimination of the organism from the carcass and safety of the product.

This is partly because of the problem of standardizing these methods, and more importantly, because much of microbial contamination is invisible. “Zero-tolerance” to visible contamination of carcass should be set as a requirement by legislation to promote spot-clean of visible areas of contamination of carcass during processing.

2.10.2.3 *Decontamination*

Several methods to reduce contamination of carcasses have been developed and tested for efficacy against *E. coli* O157:H7. These methods, which include the use of hot-water washes, steam vacuum and chemical treatments, either alone or in combination, have various degrees of success in reducing or eliminating this pathogen.

Using hot water and steam: Heat treatment, either in the form of hot-water washes or pressurized steam, is a simple way of whole-carcass decontamination to decrease high levels of the pathogen at all points on the carcass surface. The aim of heat treatment is to raise the temperature of carcass surface sufficiently high to kill *E. coli* O157:H7, along with other pathogens. One drawback of this technique is change of colour due to surface heating, which may be unacceptable to consumers. However, this effect is temporary and the carcass returns to normal colour, if the duration of treatment is short (Gannon, 1999).

Chemical treatments: The level of contamination of carcass may be reduced effectively by washing it with a variety of disinfectants added to the wash-water. Examples of such disinfectants are hydrogen peroxide (5%), trisodium phosphate (8-12%), acetic acid (2%) ozone (0.5%), fumaric acid (1%), lactic acid and acetic acid (Gannon *et al*., 1999). It was observed that temperature and number of wash-water treatments were the most important factors determining the reduction of number of organisms. Two washes, with the temperature of first wash-water at

72°C, was found to be comparatively more effective (Gannon *et al.*, 1999). Trisodium phosphate (TSP) may be used at 8% concentration (which has a pH of >12) as a postchill antimicrobial treatment as it is found to be effective in reducing *E. coli* O157:H7 in beef carcasses and potentially other food items (Feng, 2001).

Combined treatments: It was mentioned that when carcass cleaning and carcass decontamination methods were used in combination, the techniques were found to be more effective in eliminating *E. coli* O157:H7 from carcass, as compared to any one treatment alone. The following combinations were found to be equally effective in reducing *E. coli* O157: H7 levels from beef carcasses (Gannon, 1999): (i) trimming and a warm-water wash; (ii) trimming, a warm-water wash and steam pasteurization; (iii) a warm-water wash and steam pasteurization; (iv) trimming, a warm water wash, a hot lactic acid wash and steam pasteurization; and (v) a steam vacuum, a warm-water wash, a hot lactic acid wash and steam pasteurization. Farkas (1998) documented that hot water or pressurized steam, when used in combination with hot lactic acid or acetic acid solution, is an effective way of decreasing the level of *E. coli* O157:H7 contamination of beef carcass.

2.10.2.4 *Irradiation*

Ionizing radiation is mentioned to be the most effective method in eliminating *E. coli* O157:H7 from ground beef (Gannon, 1999). It has been reported that a dose range of 23 kGy of gamma irradiation is adequate to decontaminate raw meat of all food borne pathogens including *E. coli* O157:H7 (Feng, 2001). One of the advantages of irradiation is that it can be applied in prepackaged meat products, and those that are frozen (Farkas *et al*., 1998), so recontamination of the product is also less of concern. The dosage requirement, however, depends on the

temperature of the product during irradiation (e.g. fresh, refrigerated, or frozen), in addition to the physiological state of the organism (i.e. induced to acid resistance state).

However, there are certain limitations of this method, like changes in sensory attributes of meat, regulatory approval only in limited countries, and cost-effectiveness of industrial application.

2.10.2.5 *Freezing*

The conditions of freezing and thawing affect the extent of damage and death to the cells of *E. coli*, but it is clear that survival of freezing process can and does occur. Dependence on freezing to decontaminate food is therefore not a reliable practice (Bell *et al*., 1994). A study done by Dykes *et al*. (1999) to assess the survival of *E. coli* O157:H7 on frozen beef trimmings found that freezing in itself is not a reliable method of product decontamination, however, it may add margin of safety to beef trimmings contaminated with *E. coli* O157:H7, if used in combination with other methods.

2.10.3 Protection of consumers

2.10.3.1 *Proper cooking*

Cooking is the safest methods of avoiding food-borne bacterial infections, including *E. coli* O157 (Gannon, 1999). Cooking is of special importance in preventing *E. coli* O57: H7 infection as most outbreaks are related to consumption of undercooked ground beef. It is thus obvious that all ground beef and hamburger should be cooked thoroughly. Because ground beef can turn brown before *E. coli* are killed, to ensure thorough cooking digital instant-read meat thermometers can be used. Ground beef should be cooked until a thermometer inserted into several parts of the patty, including the thickest part, reads at least 160°F. It is suggested that

persons who cook ground beef without using a thermometer can decrease their risk of infection by avoiding eating ground beef patties that are still pink in the middle.

2.10.3.2 *Kitchen and personal hygiene*

Cross-contamination from contaminated meat to other foods is one of the most significant ways of getting infected from *E. coli* O157:H7. To avoid this, raw meat should be kept separate from ready-to-eat foods. Hands, counters and utensils should be washed with hot soapy water as they touch raw meat. Cooked hamburgers or ground beef should never be placed on the unwashed plates that held raw patties. Meat thermometers should be washed in between test of patties that require further cooking. Persons with diarrhoeal illness should be taken as special risks and should not be preparing meals, serving foods, or handling clean plates and utensils that are used in serving and their washing of hands with soap and water is crucial before they touch food items or utensils.

2.10.3.3 *Change in food-habit*

Only pasteurized milk, juice or cider should be consumed. Fruits and vegetables, particularly those that are eaten raw, should be washed thoroughly. Proper washing and rinsing of the produce prior to consumption may not eliminate the risk completely but have been shown to significantly decrease the bacterial populations on the produce (Feng, 2001). Children, elderly and immunocompromised people should not be allowed to eat high risk food like alfalfa sprouts, until their safety can be guaranteed. Water that has been treated with chlorine or other disinfectants should be drunk.

2.10.3.4 *Educating of the public*

A substantial proportion of *E. coli* O157:H7 infections are acquired in the home as a result of inadequate kitchen hygiene, therefore it has been suggested that these infections could be prevented through enhanced kitchen hygiene and safe food-handling at home (Mead *et al*., 1998). Introduction of safe food-handling instruction label on the meat packages is believed to be one way of improving these food-handling practices.

Since it is impossible to make the meat completely free of pathogens, improvement in consumer behaviour remains the important means of preventing *E. coli* O157:H7 infections (Mead *et al*., 1998). Since *E. coli* O157: H7 present in food is rapidly killed on cooking (Chapman *et al*., 2000), consumers should be made aware about this, e.g. by the safe food-handling label.

Consumers should be educated about the risk of eating undercooked ground beef, the importance of safe food-handling practices, and the potential for person-to-person transmission.

Food handlers infected with *E. coli* O157 should not be allowed to prepare food until they are asymptomatic and have had two negative stool cultures (CDC, 1995).

CHAPTER THREE

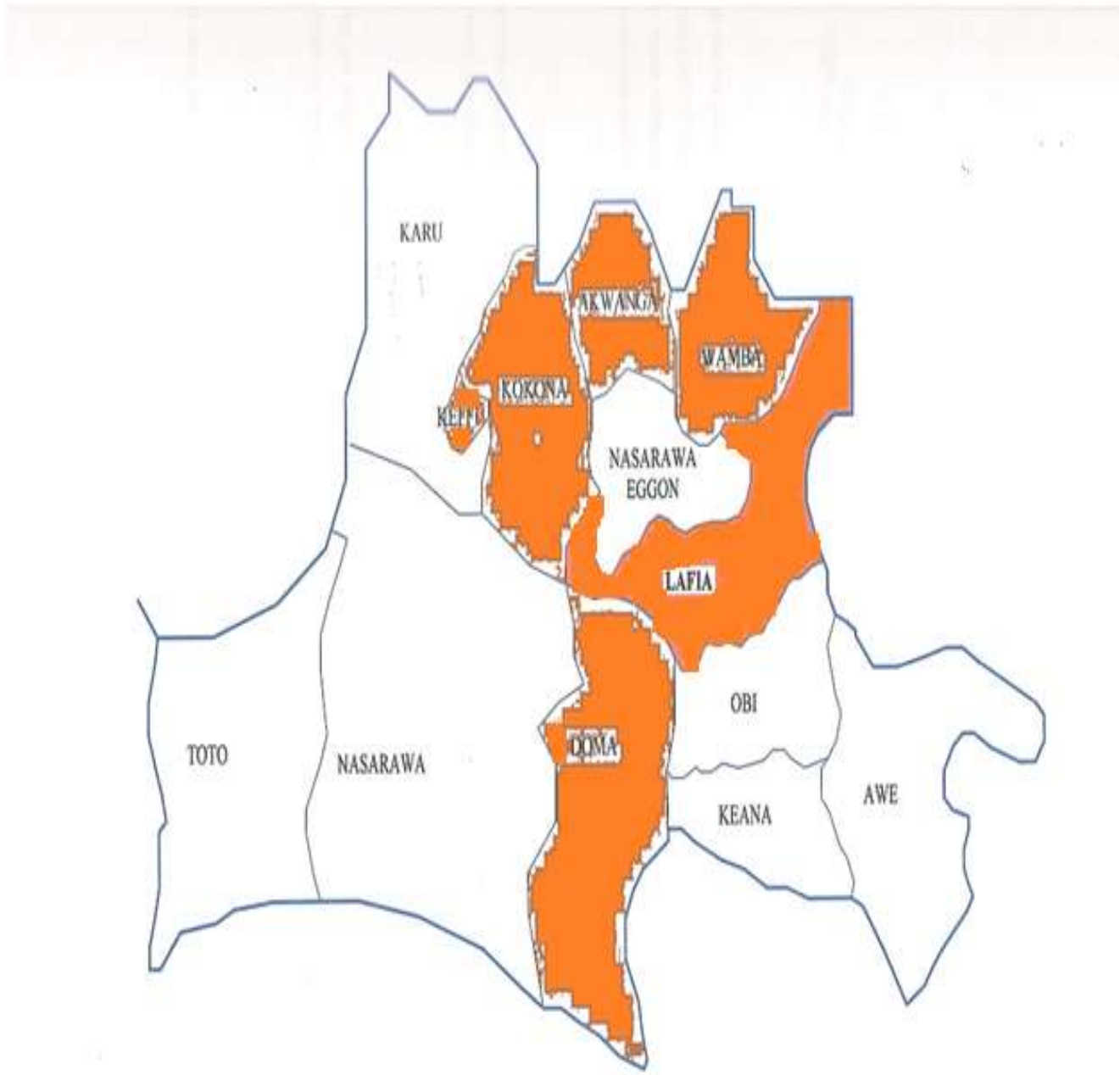
MATERIALS AND METHODS

3.1 Study Design

The study was designed as a cross-sectional study (prevalence study): an observational study that involves the selection of N sample from a larger population, and then the determination, for each sample of the simultaneous presence or absence of *E. coli* O157:H7.

3.2 Study Area

Nasarawa State falls within the Guinea Savannah zone and covers an area of about 10,470 square kilometres and between longitude 7^0 51’58’’ East and latitude 8^0 21’58’’ North in North-Central Nigeria. It is characterized by two main seasons: a rainy season (April to September), and a dry season (October to March). It is bounded in the North by Kaduna State, in the West by the Abuja Federal Capital Territory, in the South by Kogi and Benue States and in the East by Taraba and Plateau States. Nasarawa State has a projected human population of 2,040,097, having agriculture as the mainstay of its economy with the production of varieties of cash crops throughout the year (Sabo, 2011).



= Sampling sites

Figure 3.1 The map of Nasarawa State showing the sampling sites

3.3 Sample Size

The sample size was determined as described by Thrusfield, (1995)

$$n = Z^2 Pq / d^2$$

Where:

n= Sample size,

P= Prevalence (18.0% Asuke *et al*., 2012)

d= Observed absolute precision (0.05)

Z= Standard normal deviation for 95% confidence level (1.96)

q=1-p

$n = 1.96^2 \times 0.18 \times (1- 0.18) / (0.05)^2$

= 226.81

However, the sample size was increased to 420 in order to reduce sampling error.

3.4 Sample Collection and Handling

Seventy (70) *nono* samples were purchased from *nono* hawkers using convenience sampling method from the Local Government Areas selected for this study viz: Akwanga and Wamba (Nasarawa North), Lafia and Doma (Nasarawa South) and Keffi and Kokona (Nasarawa West). About 20 to 25 *nono* samples were obtained from each of the aforementioned Local Government Areas once in a week. The selection of these areas was based on cattle population and *nono* hawkers. Each sample (25ml) was collected into a sterile corked plastic tube and then labelled appropriately. All samples were placed in separate sterile plastic bags to prevent spilling and

cross contamination. Samples were stored in a cooler with ice packs and then transported to the Bacterial Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University Zaria, for Laboratory analysis within 4 to 5 hours.

3.5 Preparation of Culture Media

The media: modified Tryptone soy broth, sorbitol MacConkey agar, nutrient agar and Muller - Hinton agar were prepared by dissolving the required weight of agar (weighed in grams) in a recommended volume of distilled water in a conical flask. The instructions of the manufacturers were strictly adhered to during preparation of media as stated below. The diluted media were shaken, stirred and allowed to stand for at least thirty minutes before they were heated. After heating, they were autoclaved at 121^0C for 15 minutes. Supplements were added after the media had cooled to about 55^0C and where there was no supplementation; media were dispensed into sterilized Petri dishes after they were cooled to about 55°C. They were allowed to solidify and then stored at 4^0C for use within a day or two.

3.6 Isolation of *E. coli* O157:H7

The steps for the isolation of *E. coli* O157:H7 were conducted according to the isolation procedure of ISO (2003). These steps included: Enrichment, Selective plating, biochemical characterization and serological confirmation by latex agglutination as shown below:

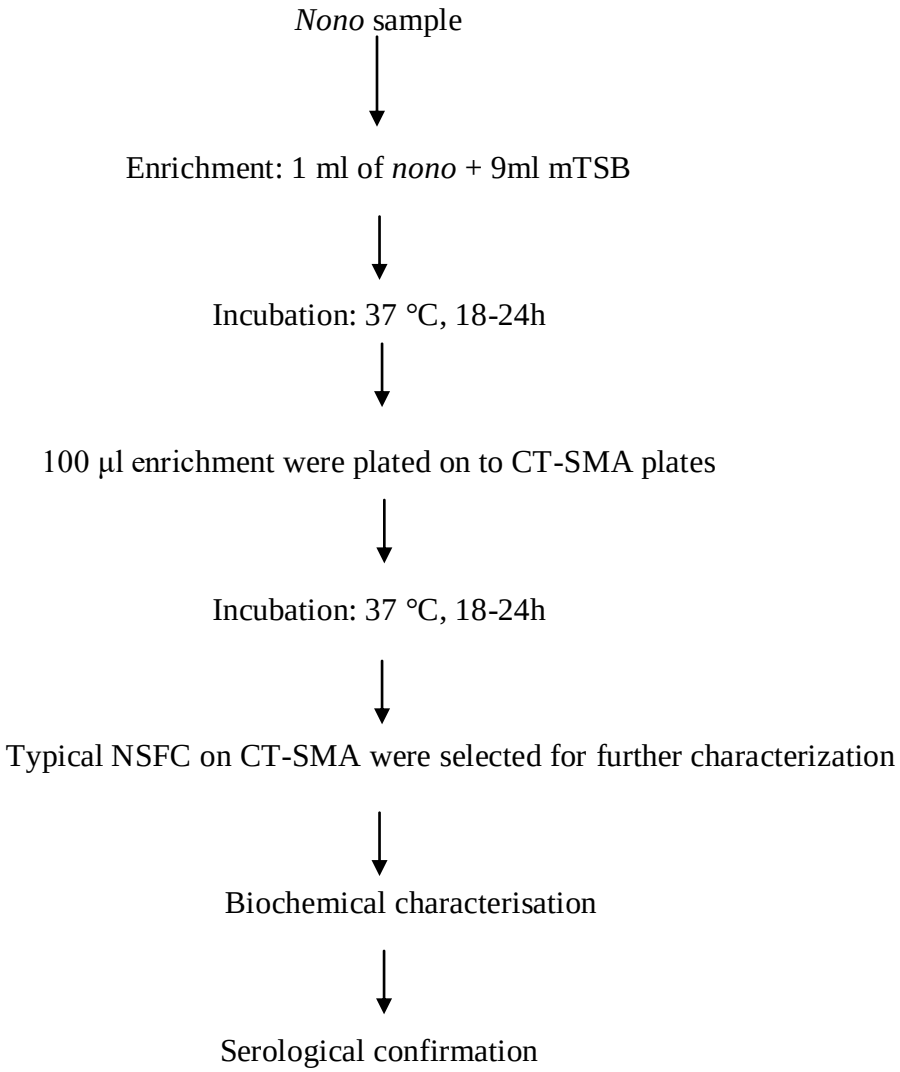


Figure 3.2 Flow chart of the isolation of *E. coli* O157:H7 from *nono*.

3.6.1 Enrichment

One millilitre of the *nono* sample was directly added to 9ml modified Tryptone soy broth supplemented by novobiocin (mTSB+N). The inoculated broths were incubated at 37^{o}C for 24hours.

3.6.2 Selective plating and identification of *E. coli* O157:H7 colonies

A loopful of the enrichment broth was streaked onto Telluritte-Cefixime Sorbitol MacConkey agar (CT-SMA) plate and incubated at 37^{o}C for 24 hours.

A typical *E. coli* O157:H7 appeared as a non-sorbitol fermenter colony (NSFC) which is characterized as having a slightly transparent, almost colourless with a weak pale brownish appearance. Individual discrete colonies from the CT-SMAC agar plates were picked and sub-cultured onto nutrient agar slants and incubated at 37^{o}C for 24hours. This was then refrigerated for further biochemical and serological analysis.

3.6.3 Gram staining

The plates with non-sorbitol fermenting colonies (NSFC), which are usually colourless colonies, were noted, after which discrete colonies were randomly selected and then examined for the presence of gram-negative rods using Gram staining technique (Prescott *et al*., 2005).

3.6.4 Biochemical test

The isolates were characterized biochemically using Microbact 12E (MB1130A^{+}, Oxoid) according to the manufacturer's instruction. Identification was done following a series of biochemical tests: Voges-Proskauer reactions, indole, citrate, urea hydrolysis, hydrolysis of O-nitrophenyl-β-d-galactopyranoside(ONPG), production of indolepyruvate by deamination of tryptophan, sugar (glucose, mannitol and xylose) fermentation, nitrate reduction, H_2S production, lysine and ornithine decarboxylase.

3.6.5 Serological test

E. coli positive colonies were serologically confirmed by using *E. coli* O157:H7 latex agglutinations assay (R30959601, Oxoid), containing latex particles coated with antibodies specific for *E. coli* O157 and *E. coli* H7 antigen. Isolates were tested separately with anti-O157, and H7 antisera. Identification of *E. coli* O157:H7 was carried out following the manufacturer's instruction, hence colonies that agglutinated to the separate antisera were considered to be *E. coli* O157:H7.

3.7 Antimicrobial Susceptibility Test

The isolates were tested for antimicrobial susceptibility, using the agar disc diffusion method of Kirby-Bauer, (1966). The following antibiotics (Oxoid) were used: Penicillin (10 units), Gentamicin (10µg), Ciprofloxacin (5µg), Streptomycin (10µg), Amoxycillin (25µg),

Tetracycline (30µg), Chloramphenicol (30µg), Oxacillin (5µg), Erythromycin (5µg) and Sulphamethoxazole/Trimethoprim (25µg).

The isolates were spread onto Muller-Hinton agar plate and the antibiotic impregnated discs were applied onto the inoculated plates using sterile forceps. The plates were then incubated at 37^{o}C for 24hrs, after which clear zones of inhibition for each antibiotic were measured using transparent ruler. The results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2007).

3.8 Data Analysis

The results obtained were presented using tables. Chi-square was used to analyzed data with the aid of the statistical package for social science (SPSS) version 17.0 (SPSS Inc. Chicago IL, USA).

CHAPTER FOUR

RESULTS

4.1 Occurrence of *E. coli* O157:H7

The prevalence of *E. coli* O157:H7 in locally fermented milk (*nono*) samples obtained from various studied Area are resented in Table 4.1. Out of the 420 *nono* samples examined, 19 (4.5%) were found to be contaminated with *E. coli* O157:H7. The percentages of occurrence of this pathogen across the selected Local Government Areas in Nasarawa States were: Akwanga 5.7%, Wamba 5.7%, Lafia 2.9%, Doma 5.7%, Keffi 2.9% and Kokona 4.3%. The overall

frequency of occurrence of *E. coli* O157:H7 is between 2.9% to 5.7% across the 6 Local Government Areas (Table 4.1).

Table 4.2 gives the prevalence of *E. coli* O157:H7 across the senatorial zones of Nasarawa State thus: Nasarawa North 5.7%, Nasarawa West 3.6% and Nasarawa South 4.3%.

Table 4.3 shows the occurrence of *E. coli* O157:H7 with respect to the market sites. *Nono* sold in Market areas were found to harbour *E. coli* O157:H7 with a prevalence of 5.0%, while those sold on Streets and in Schools had rates of 3.2% and 4.2% respectively.

4.2 Antimicrobial Susceptibility of *E. coli* O157:H7

Table 4.4 shows the antimicrobial susceptibility patterns of the 19 *E. coli* O157:H7 isolated from locally fermented milk (*nono*) samples, tested against 10 antibiotics. Seventeen (89.5%) of the isolates were sensitive to gentamicin, 15 (78.9%) to ciprofloxacin, 8 (42.1%) to streptomycin, 3 (15.8%) to oxacillin and sulphamethoxazole/trimethoprim, 1 (5.3%) to chloramphenicol and amoxicillin.

Antibiotic susceptibility profiles showed that virtually all the isolates were resistant to one or multiple antibiotics. However, 19 (100%) of the *E. coli* O157:H7 isolates were resistant to penicillin and tetracycline, 18 (94.7%) to erythromycin, 16 (84.2%) to amoxicillin, oxacillin and sulphamethoxazole/trimethoprim, 13 (68.4%) to chloramphenicol, 8 (42.1%) to streptomycin and none was resistant to ciprofloxacin and gentamicin (Table 4.4). Gentamicin, ciprofloxacin and streptomycin were the most effective antibiotics whereas penicillin, tetracycline and erythromycin were the least effective.

The antimicrobial resistance patterns are shown in Table 4.5. The most common patterns were penicillin-tetracycline-chloramphenicol-amoxycillin-erythromycin-oxacillin-sulphamethoxazole/trimethoprim with a frequency of 7 (36.8%), followed by penicillin-streptomycin-tetracycline-chloramphenicol-amoxycillin-erythromycin-oxacillin-sulphamethoxazole/trimethoprim 3 (10.5%), penicillin-strepromycin-tetracycline-amoxycillin-erythromycin-oxacillin-sulphamethoxazole/trimethoprim 2 (10.5%) and penicillin-tetracycline-amoxycillin-erythromycin-oxacillin-sulphamethoxazole/trimethoprim 2 (10.5%). Penicillin and tetracycline resistance were most common among the various patterns observed. The highest levels of multidrug resistance observed were in isolates from Wamba, Doma, Kokona and Keffi Local Government Areas respectively.

Table 4.1. Frequency of occurrence of *E. coli* O157: H7 in Locally-Fermented Milk (*Nono*) Sold in 6 local government areas of Nasarawa State.

LGA	No of Samples Tested	No. (%) Positives

Akwanga	70	4 (5.7)
Wamba	70	4 (5.7)
Lafia	70	2 (2.9)
Doma	70	4 (5.7)
Keffi	70	2 (2.9)
Kokona	70	3 (4.3)
Total	420	19 (4.5)

Table 4.2. Prevalence of *E. coli* O157: H7 in locally fermented milk (*nono*) sold in three senatorial zones of Nasarawa State.

Senatorial zone	No. (%) of sample	No. (%) positive
Nasarawa North	140	8 (5.7)
Nasarawa South	140	6 (4.3)
Nasarawa West	140	5 (3.6)
Total	420	19 (4.5)

Table 4.3. Occurrence of *E. coli* O157: H7 in locally fermented milk (*nono*) with respect to sites of sampling.

Site	No. Examined	No. (%) contaminated with *E. coli* O157:H7
Market	302	15(5.0)
Street	93	3(3.2)
School	25	1(4.2)
Total	420	19 (4.5)

Table 4.4. Antimicrobial susceptibility of 19 *E .coli* O157:H7 isolates from locally fermented milk (*nono*) sold in Nasarawa State.

		Susceptibility		
		R	I	S
Antibiotic	Concentration (µg)	No. (%)	No. (%)	No. (%)
Penicillin	10	19 (100.0)	0 (0.0)	0 (0.0)
Ciprofloxacin	5	0 (0.0)	4 (21.1)	15 (78.9)
Gentamicin	10	0 (0.0)	2 (10.5)	17 (89.5)
Streptomycin	10	8 (42.1)	3 (15.8)	8 (42.1)
Tetracycline	30	19 (100.0)	0 (0.0)	0 (0.0)
Chloramphenicol	30	13 (68.4)	5 (26.3)	1 (5.3)
Amoxicillin	25	16 (84.2)	2 (10.5)	1 (5.3)
Erythromycin	5	18 (94.7)	1 (5.3)	0 (0.0)
Oxacillin	5	16 (84.2)	0 (0.0)	3 (15.8)
Sulphamethoxazole/ Trimethoprim	25	16 (84.2)	0 (0.0)	3 (15.8)

Table 4.5. Antimicrobial resistance patterns of the 19 isolates of *E. coli* O157:H7.

S/N	Resistance Pattern*	No. (%) of Isolates Showing Patterns	LGA**
1	Pen, Tet, Chl, Ery	1 (5.3)	Wa
2	Pen, Str, Tet, Sul	1 (5.3)	Ak
3	Pen, Str, Tet, Ery, Sul	1 (5.3)	La
4	Pen, Tet, Amo, Ery,Oxa, Sul	2 (10.5)	Ke, Ko
5	Pen, Tet,Chl, Amo, Ery,Oxa	1 (5.3)	Wa
6	Pen, Tet, Chl, Amo, Ery, Oxa, Sul	7 (36.8)	Wm, Do, ko, ko
7	Pen, Str, Tet, Amo, Ery, Oxa, Sul	2 (10.5)	Do, Ak
8	Pen, Str, Tet, Chl, Amo, Ery, Oxy	1 (5.3)	Ak
9	Pen, Str, Tet, Chl, Amo, Ery,Oxa, Sul	3 (15.8)	Do, Ak, La

Symbols: *Pen, penicillin; Tet, tetracycline; Chl, chloramphenicol; Cip, ciprofloxacin; Gen, gentamicin; Str, streptomycin; Ery, Erythromycin; Amo, Amoxycillin; Oxa, oxacillin; Sul, sulphamethoxazole/trimethoprim

** Wa, Wamba; Ak, Akwanga; La, Lafia; Ke, Keffi; Ko, Kokona and Do, Doma.

CHAPTER FIVE

DISCUSSION

In some developed countries, *E. coli* O157:H7 has been detected in raw milk and milk products (Armstrong *et al*., 1996). Likewise the results obtained from this research revealed that this organism was present in locally fermented milk (*nono*) marketed in Nasarawa State, Nigeria. Apart from detecting *E. coli* O157:H7 from bovine milk, it has also been documented that it is a vehicle for *E. coli* O157:H7 infection (Borczyk *et al*., 1987; Wells *et al*., 1999; Ostroff *et al*., 1989; Coia *et al*., 1998), and even in Nigeria in the recent years (Asuke *et al*., 2012; Itelina *et al*., 2010; Ogbonna, 2011).

Serotype O157:H7 strains of *E. coli* remains a serious threat to public health and an appreciable economic burden to the food industry. Numerous studies have shown that cattle and food produced there from, particularly ground beef are significant sources and vehicles of transmission of this pathogen (Byrne *et al*., 2002).

The percentage occurrence of *E. coli* O157:H7 in locally fermented milk (*nono*) in Nasarawa State recorded in this study is 4.5%. This is higher than the prevalence in Plateau State, Nigeria, where 5 (0.71%) out of 350 *nono* samples were contaminated with *E. coli* O157:H7 (Itelima *et*

al., 2010). The occurrence rate recovered in this study is also higher than the data reported in raw milk from the United States of America in which out of the 1, 021 bovine milk samples examined, 20 (2.0%) were positive for *E. coli* O157:H7 (Armstrong *et al*., 1996). A lower occurrence rate of *E. coli* O157:H7 (O.5%) in traditional dairy products was recorded from a study in Iran (Rahimi *et al*., 2012) as compared with the result obtained from this study.

E. coli O157:H7 has also been isolated from yoghurt which is also a fermented milk product (Morgan *et al*., 1988). These authors reported that the occurrence rate was 5% in some yoghurt samples and some samples contained the organism at levels of ≤ 10 cells per millilitre. They suspected that contamination of fermented milk samples may have come from raw milk used for its production or from handlers due to poor processing. The significance of the isolation of *E. coli* O157:H7 from local milk product (*nono*) in the present study is of general public health concern because many people in Nigeria, mostly in the northern States consume this product. Thus, the product may be one of the major vehicles for *E. coli* O157:H7 transmission from cattle to man.

Studies have shown that raw milk usually has a higher incidence of *E. coli* O157:H7 than fermented milk products (Wilson *et al*., 1998; Heuvelink *et al*., 1998). This is certainly a consequence of higher pH values observed in raw milk than in fermented milk such as *nono*. Buchanan and Klawitter (1992) reported that the growth of *E. coli* O157:H7 decline rapidly at low pH values. Shehu and Lamido (1994) also explained the reason for the acidic nature of *nono*. They reported that during the production of *nono* by the Fulani milk producers in Northern Nigeria, they usually add sour white extract of the seed of baobab tree. It is suspected that this extract contributes in further lowering the pH of the fermented milk product. It is generally believed that the extract is added in order to retain the flavour of the *nono* after it has been

diluted with water for economic reasons. It is also believed that in this way the consumer may not be able to distinguish between the original colour and the flavour of the fermented product and that of the diluted product.

There was no significant difference (P>0.05) in the occurrence of *E. coli* O157:H7 isolated from *nono* samples with respect to the various Local Government Areas, indicating that the milk produced and marketed in these areas may have similar microbial quality. This may be due to the fact that similar handling procedures are employed during milking process and during the production of fermented milk (*nono*) in the various areas.

The relatively higher occurrence of *E. coli* O157:H7 in locally fermented milk (*nono*) in study area may be attributed to lack of effective sanitary precautions and less careful or unhygienic handling procedures during milking process and *nono* production. The use of traditional milking methods also expose milk to pathogenic bacteria found in cow udders and probably, on the hands of the milkers who might have come in contact with faeces of the cows. The unhygienic environmental conditions where *nono* is marketed may also contribute to its contamination. The use of more sensitive assay such as enrichment broth and selective media for the isolation of *E. coli* O157:H7 in this study might have helped in obtaining a higher occurrence of the organism. In a study in USA by Clark *et al*. (1989), *E. coli* O157:H7 was not isolated from any of the milk samples examined. These authors suggested that the absence of the organism in milk was as a result of the use of an assay which was not sensitive to detect *E. coli* O157:H7.

The antimicrobial sensitivity tests showed a high prevalence of resistance by the *E. coli* O157:H7 isolates tested. The development of antimicrobial resistance by the bacteria to these drugs poses a major challenge in both human and animal medicine because these drugs are commonly used

in the treatment of human patients and in veterinary practice. Uncontrolled usage of antibiotics in treatment of animals and their incorporation in animal feeds has been suspected to account significantly for the increase in antibiotic resistance in human bacterial isolates (WHO, 2000; Galland *et al*., 2001).

All the isolates (100%) tested were resistant to Penicillin and tetracycline. This is in agreement with the finding of Olatoye (2010), who recorded a high level (91.4%) of resistance to tetracycline by isolates of *E. coli* O157:H7. Al Haj *et al.* (2007) also observed high resistance (81.4%) of *E. coli* O157:H7 to tetracycline, Shitandi and Sternesjö (2001) also obtained high (72%) and (57.9%) resistance by *E. coli* O157:H7 to penicillin and tetracycline. O'Brien (1987) also reported high resistance (72%) to tetracycline. The high level of resistance by *E. coli* O157:H7 to tetracycline obtained in this study may be due to the fact that it is the most commonly available antibiotic used as growth promoter and routine chemoprophylaxis among livestock in Nigeria (Olatoye, 2010). This is worrisome considering that tetracycline is a first line drug in Nigeria, and as in most developing countries, people with gastrointestinal infections readily purchase it across the counter for self-medication (Chigor *et al*., 2010). Penicillin resistance as obtained from this study may be as a result of the frequent usage of this antibiotic in treating diseases in cattle (Byrne *et al*., 2002). According Okeke *et al*. (1995) penicillin and tetracycline are known to be extensively used in developing countries.

High rate of resistance to sulphamethoxazole/trimethoprim (84.2%) is in agreement with the previous work by Shroeder *et al*. (2002), who reported that among 189 *E. coli* O157:H7 isolates recovered from various sources between 1985 and 2000, 19 (10%) were resistant to this antibiotic, which was the highest among the antibiotics tested. This antimicrobial is commonly used to treat respiratory infections, diarrhoea, mastitis, and other infections in beef and dairy

cattle (Shroeder *et al*., 2001). Resistance was found to be relatively low to streptomycin. This probably may be because of less exposure to the antibiotic and the fact that it is usually administered intravenously thereby restricting indiscriminate use (Cheesbrough, 2000). This shows that streptomycin can be used as an antibiotic of choice against *E. coli* O157:H7 infections, except for its serious side effect (Prescott *et al*., 2005)

The high prevalence of resistance of *E. coli* O157:H7 isolates to erythromycin, oxacillin, amoxicillin and chloramphenicol is of importance from the view point of medical and veterinary practice in Nigeria. This could be a reflection of use and misuse of these antibiotics in the study area. This finding is not surprising because outside the hospital environment the general population have easy access to various antibiotics at any drug store without any prescription from a medical practitioner.

In this study, all the *E. coli* O157:H7 isolates tested showed multidrug resistance to the antibiotics tested. This result is in agreement with the findings by other researchers, who reported multidrug resistance among *E. coli* O157:H7 isolates (Kim *et al*., 1994; **Schroeder *et al*., 2002**). Various isolates were simultaneously resistant to 4, 5, 6, 7 and 8 of the antibiotics tested. Isolates from Doma, Akwanga and Lafia showed higher frequencies of multidrug resistance. Multiple antimicrobial resistance in *E. coli* O157:H7 isolates may partly result from the spread of genetic elements including plasmids, transposons, and integrons (Zhoa *et al*., 2001) that may confer resistance to numerous antimicrobials. According to Aarestrup (1995) and Levin *et al*. (1997), multiple resistances capable of regional dissemination can emerge as a result of antimicrobial selection pressure in either livestock or humans. Evidence has been found which indicates that resistant strains of pathogens can be transmitted to humans through food (Oosterom, 1991; Khachatourians, 1998).

This high multidrug resistance among *E. coli* O157:H7 isolates as seen in this study is quite alarming. The selection and spread of resistant organisms in developing countries, which can often be traced to complex socioeconomic and behavioural antecedents, has contributed to the escalating problem of antibiotic resistance worldwide (Okeke *et al*., 1995).

Results from this study indicate that ciprofloxacin (fluoroquinolone) and gentamicin (aminoglycoside) may be the drugs of choice for *E. coli* O157:H7, since all of the isolates were sensitive to them. This shows the effectiveness of the fluoroquinolones and aminoglycosides, and is in agreement with the finding of Scheld (2003).

The public health significance of these findings is that antimicrobial resistant *E. coli* O157:H7 from *nono* (or dairy animals) may spread to humans via the food chain, such as consumption of *nono*, contact through occupational exposure, or waste run-off from *nono* production facilities to the neighbourhood. Indiscriminate use of antimicrobials among livestock producers and marketers in Nigeria could also be responsible for the resistance patterns obtained in this study.

The development of antimicrobial resistance by the *E. coli* O157:H7 isolates to these drugs poses a major challenge in both human and animal medicine because these drugs are commonly used in the treatment of human patients and in veterinary practice (WHO, 2000; Galland *et al*., 2001). Shedding of the resistant bacteria into the environment by cattle may lead to a widespread dissemination of antibiotic resistant genes to the resident bacteria in the environment (Callaway *et al*., 2003; Mashood, *et al*., 2006).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Escherichia coli O157:H7 is a newly emerging pathogen frequently associated with the consumption of food of bovine origin. Severe and life-threatening human diseases caused by *E. coli* O157:H7 strains have been reported throughout the world. The severity of the infections caused by this food borne pathogen in the young and the elderly has had a tremendous impact on human health and the food industry. The present study evaluated the occurrence of *E. coli* O157:H7 in locally fermented milk (*nono*) sold under market conditions in Nasarawa State and the patterns of their antibiotic susceptibility. A total of four hundred and twenty *nono* samples were collected across Nasarawa State and subjected to cultural, biochemical and serological tests

for detection of *E. coli* O157:H7. The isolates were subjected to antimicrobial susceptibility testing using the agar disc diffusion technique. *E. coli* O157:H7 were isolated from 19 (4.5%) of 420 *nono* samples examined. Among the samples examined, the highest prevalence (5.7%) was recorded in samples obtained from Akwanga, Wamba and Doma Local Government Areas, while Lafia and Keffi had the least prevalence rate (2.9%). With respect to the senatorial zones, Nasarawa North had the highest prevalence rate of 5.7% while the Southern zone had the least (3.6%). There was no significant difference ($P>0.05$) in the occurrence of *E. coli* O157:H7 isolated from *nono* samples with respect to the various Local Government Areas. Antibiotic susceptibility profiles showed that all the isolates were resistant to multiple antibiotics, resulting in nine different resistance patterns. Nineteen (100%) of the isolates were resistant to penicillin and tetracycline, 18 (94.7%) to erythromycin, 16 (84.2%) to amoxicillin, oxacillin and sulphamethoxazole/trimethoprim, 13 (68.4%) to chloramphenicol and 8 (42.1%) to streptomycin. Fifteen (78.9%) and 17 (89.5%) of the isolates were sensitive to ciprofloxacin and gentamicin. The predominant antimicrobial resistance pattern was penicillin-tetracycline-chloramphenicol-amoxycilin-erythromycin-oxacillin-sulphamethoxazole/ trimethoprim with the occurrence rate of 36.8% among the 19 isolates tested. *Nono* consumption has potential health risks to consumers not just in Nasarawa State but possibly to the nation at large.

6.2 Conclusion

Generally, the results obtained from this study showed that locally fermented milk (*nono*) available to consumers in Nasarawa State may be contaminated with *E. coli* O157:H7, which may probably lead to human infections as have been reported in other countries. Furthermore, the detection of *E. coli* O157:H7 in locally fermented milk (*nono*) produced in Nasarawa State, Nigeria, suggests that *nono* consumption has potential health risks to consumers.

The antibiograms of *E. coli* O157:H7 isolates showed a high prevalence of resistance to most of the antibiotics used. Although, antibiotic treatment is not recommended for *E. coli* O157:H7 infections in humans or food animals, yet virtually all the isolates were resistant to one or more antibiotics. The data suggest that selection pressure imposed by the use of these antibiotics whether therapeutically in human and veterinary medicine or as prophylaxis in the animal production, may be a key driving force in the selection of antimicrobial resistance in *E. coli* O157:H7.

5.3 Recommendations

1. The practice of preparation and distribution of *nono* in open calabash should be discouraged because it exposes the product to contamination.
2. High level of hygiene should be observed during milking and production of *nono* so as to avoid contamination.
3. There is a need to legislate and enforce laws to limit the prescription and dispensing of antibiotics and other drugs to only qualified professionals. Education of the public on the dangers of indiscriminate purchase and use of drugs is also imperative.
4. There is a need for continuous surveillance of antimicrobial resistance trends particularly among organisms resident in the gastrointestinal tract of farm-animals which are implicated in infectious diseases in humans.
5. The fluoroquinolones and aminoglycosides should be preserved by limiting their use.
6. Due to limitation of data about the occurrence of *E. coli* O157:H7 in locally fermented milk (*nono*), further studies should be directed to this kind of foods in Nasarawa State.

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APPENDICES

Appendix I: Composition of Media

Modified tryptone soya broth (CM0989; Oxoid, Basingstoke, UK)

Typical formula (g/l)

Pancreatic digest of casein	17.0
Papaic digest of soy bean meal	3.0

Sodium chloride	5.0
Di-basic potassium hydrogen phosphate	2.5
Glucose	2.5
Bile salts	1.5

Dissolve 16.5 g in 500ml of distilled water and distribute into final containers. Sterilize by autoclaving at 121^{o}C for 15 minutes.

Sorbitol MacConkey Agar (SMAC**,** CMS0813; Oxoid, Basingstoke, UK)

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
Agar	15.0

Suspend 51.5 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121 ^{o}C for 15 minutes.

Nutrient agar (CM 0003; Oxoid, Basingstoke, UK)

Typical formula (g/l)

'Lab-Lemco'powder	1.0

Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0

Suspend 28 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 12^{o}C for 15 minutes.

Appendix II. Table of substrate and reactions of Microbact 12E(Oxoid, Basingstoke, UK)

	Reaction colours

Well No.	Designation	Symbol	Negative	Positive
1	Lysine	LYS	Yellow	Blue-green
2	Ornithine	ORN	Yellow-green	Blue
3	H_2S	H2S	Straw colour	Black
4	Glucose	GLU	Blue-green	Yellow
5	Mannitol	MAN	Blue-green	Yellow
6	Xylose	XYL	Blue-green	Yellow
7	ONPG	ONP	Colourless	Yellow
8	Indole	IDN	Colourless	Yellow
9	Urease	URE	Straw colour	Pink-red
10	Voges Proskauer	VP	Straw colour	Pink-red
11	Citrate	CIT	Green	Blue
12	TDA	TDA	Straw colour	Cherry-red

APPENDIX III. Chart showing the expected result on Microbact System after 18-24hour incubation

Well No.	Symbol	*Escherichia coli* (ATCC 259922)
1	LYS	+
2	ORN	+
3	H2S	_
4	GLU	+
5	MAN	+
6	XYL	+
7	ONP	+
8	IDN	+
9	URE	_
10	VP	_
11	CIT	_
12	TDA	_

Appendix IV: Antimicrobial inhibition zone size interpretative chart

			Diameter of zones of inhibition in mm		
Antimicrobial agent	Symb	Disk content (µg)	Resistant mm or less	Interme-diate (mm)	Sensitive (mm)
Amoxicillin	Amp	25	13	14-16	17
Erythromycin	Cef	5	12	13-14	15
Chloramphenicol	Chl	30	12	13-17	18
Ciprofloxacin	Cip	5	15	16-20	21
Sulphamethoxaz ole/Trimethoprm	Cot	25	12	13-16	17
Gentamycin	Gen	10	12	13-14	15
Oxacillin	Pef	5	13	14-16	17
Penicillin	Ofx	10	13	14-16	17
Streptomycin	S	10	11	12-14	15
Tetracycline	Aug	30	11	12-14	15

Adapted from Clinical and Laboratory Standard Institute (CLSI) Zone Diametre Interpretive Standard (2007).

Based on results obtained using Mueller-Hinton Agar and *E. coli* (ATCC 25922) as positive control Symb = Symbol

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Appendix V. The following table shows how the results obtained with the *E. coli* O157 Latex Reagents and the *E. coli* O157 Positive Control should be interpreted

O157 Latex Reagent	Negative Contol Latext Reagent	Remarks
+	–	Kit performance is satisfactory
+	–	Potency is too low. Discard reagents.
+	–	Autoagglutination: Discard reagents.

Appendix VI. Agglutination of latex reagents with test specimen is interpretation chart

O157 Latex Reagent	Negative Contol Latext Reagent	Remarks
+	_	Presumptive for *E. coli* O157
+	+	Autoagglutinating or cross-Reacting strain present. Perform further test to rule out *E. coli* O157
-	not done	indicate absence of *E. coli* O157
Stringy or mucoid Appearance	not done	uninterpretable. Make fresh suspension of colonies in saline and allow clumps to settle out. Retest supernatant.

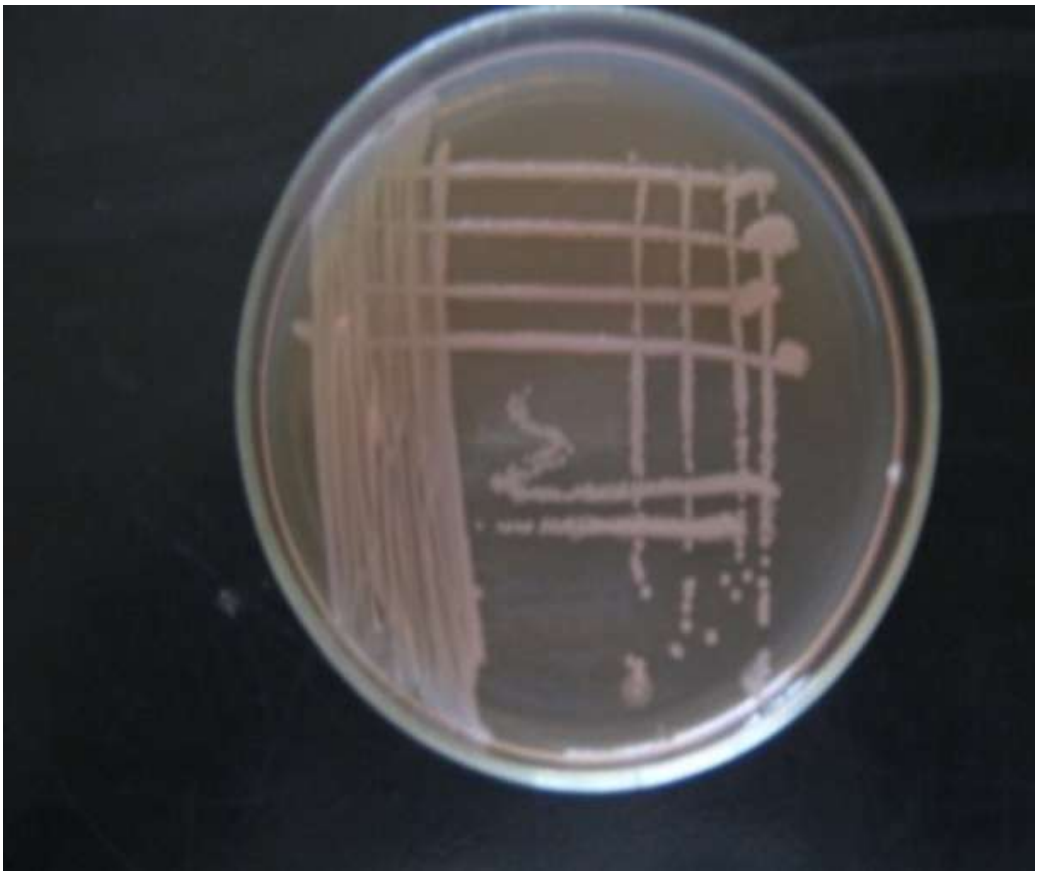

Plate I *E. coli* O157:H7 on sorbitol MacConkey (SMAC) agar

Colourless colonies presumptive of *E. coli* O157:H7

A

B

Plate II A and B showing substrate reaction of *E. coli* on Microbact 12E

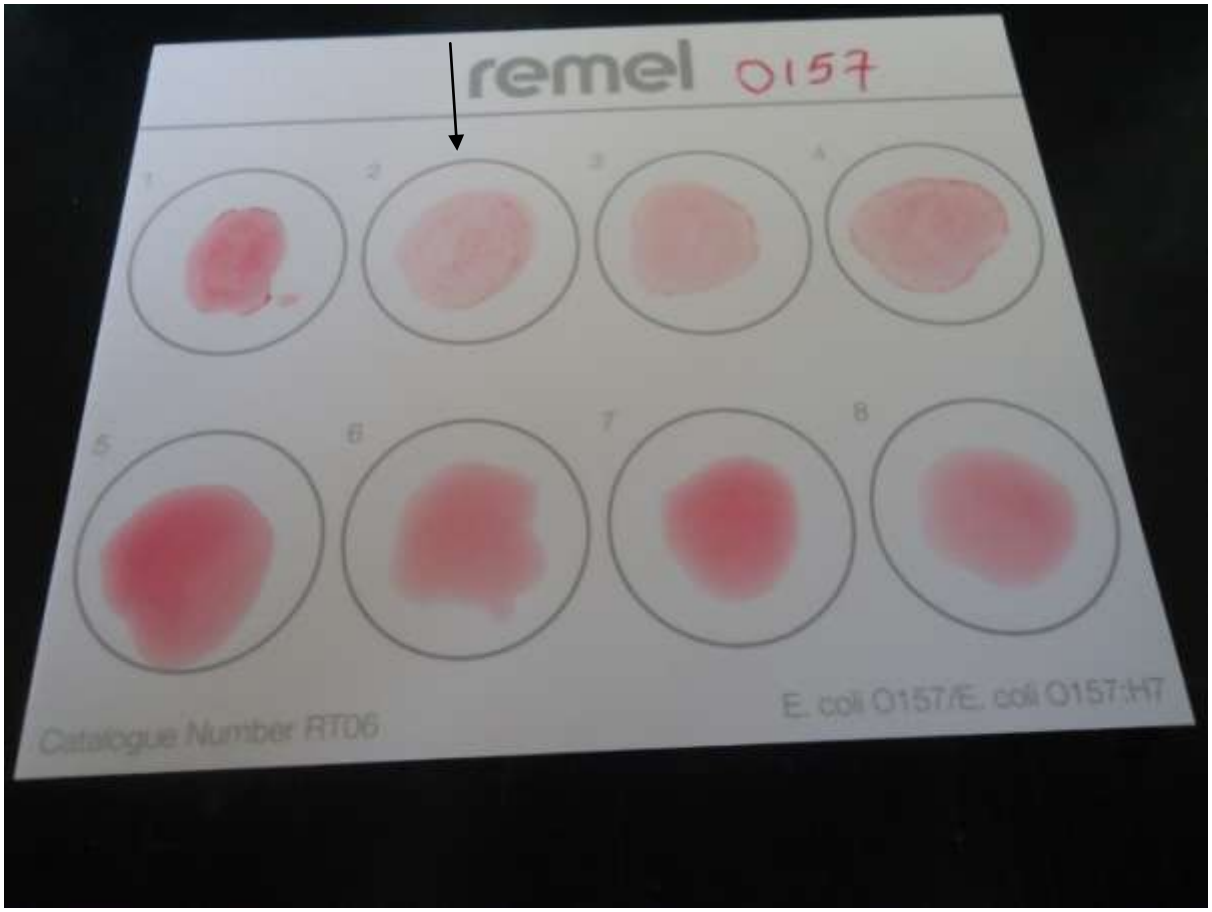

Plate III Latex agglutinations of *E. coli* O157

Plate IV Latex agglutinations of *E. coli* H7

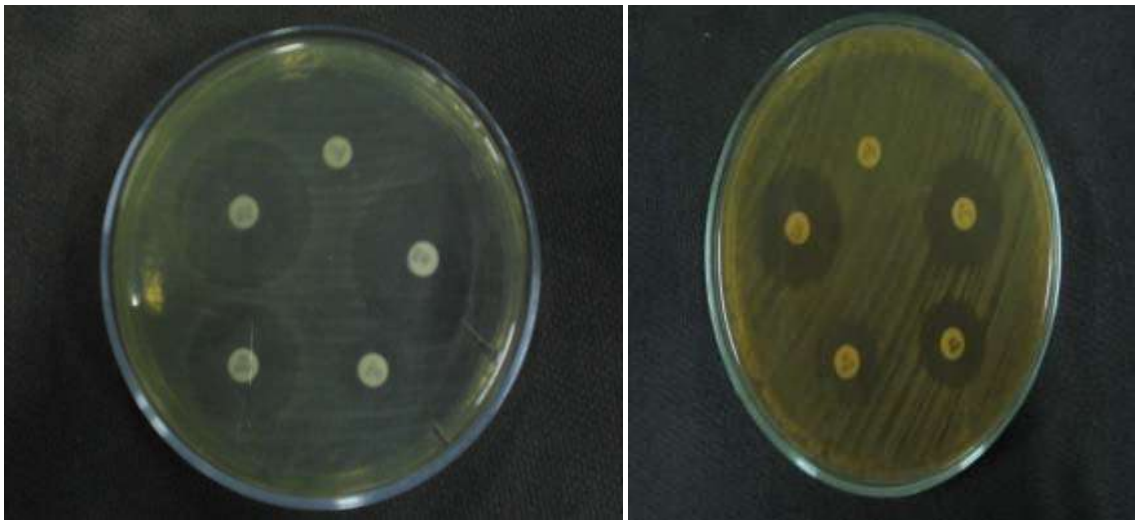

Plate VII Antimicrobial susceptibility pattern of *E. coli* O157:H7