ANTIBIOTICS SUSCEPTIBILITY STUDIES OF SOME BACTERIAL ISOLATES FROMPACKAGED MILK MARKETED IN ZARIA, NIGERIA

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AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

NOVEMBER, 2012

DECLARATION

I hereby declare that the work in this thesis titled "Antibiotics susceptibility of some bacterial isolates frompackaged milk marketed in Zaria, Nigeria" was performed by me in the Department of Pharmaceutics and Pharmaceutical Microbiology, under the supervision of Dr. Mrs. G.O Adeshina and Prof. J.O.Ehinmidu. 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.

CERTIFICATION

This thesis titled "Antibiotics susceptibility studies of some bacterial isolates frompackaged milk marketed in Zaria, Nigeria" by **UmofiaInimfon Damian** meets the regulations governing the award of the degree of Masters of Science Pharmaceutical Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Prof. A.A. Joshua Dean, School of Postgraduate Studies	Signature	Date

DEDICATION

This work is dedicated to my two favorite educators, my dad and mum for their support and encouragement throughout all my life that made me who I am. You are the best parent ever.

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I wish to express my sincere appreciation to the chairman of my supervisory committee, Dr. Mrs. G. O. Adeshina for her invaluable guidance, patience and support throughout the course of this work. I am also indebted to Prof. J. O. Ehinmidu, member of my supervisory committee for his instructions, ideas, suggestions and constructive criticism in making this work a success.

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ABSTRACT

Milk contamination with antibiotic resistant bacteria can be a major threat to public health, as the antibiotic resistant determinants can be transferred to other pathogenic bacteria potentially compromising the treatment of severe bacterial infections. This study was conducted to investigate the antibiotics susceptibility of bacterial isolates frompackaged milks marketed in Zaria. Two hundred packaged milk samples were bought from five locations (forty samples from each) in Zaria. Isolation and identification of the bacteria specieswere carried out using standard microbiological procedures. Antibiotics susceptibility of the isolates was determined using a panel of 12 antibiotics by disc diffusion method following Clinical Laboratory Standard Institute guidelines. Minimum Inhibitory Concentration (M.I.C.)was determined using agar plate dilution method.Conjugative studies were carried out with multiple antibiotics resistant isolates from milk samples. The resistant isolates were subjected to DNA isolation and agarose gel electrophoresis. The result obtained showed that the major contaminants of milk products analysed were *Pseudomonas spp*closely followed by *Enterobacterspp*and *Escherichia coli* and the overall contamination level of bacterial isolates in this study was 76.5%. One hundred and fifty-three bacterial isolates were identified from the milk sample, 27.5% were obtained from the first brand of milk sample, 21.6% from the second brand, 12.4% from the third brand and 38.6% from the fourth brand of milk samples. Susceptibility result showedthat high percentage of isolates were resistant to cloxacillin (99.35%), erythromycin (98%), amoxicillin (83.01%), chloramphenicol (83%) and tetracycline (81.7%) but were however susceptible to ofloxacin (99.3%) and gentamicin (83%). Multiple antibiotics resistance indices (MARI) showed that bacterial isolates from the studied packaged milk samples were multi-resistant with MARI ranging from 0.2 to 1.0. Out of ninety enterobacteriaceae studied, 93.3% of the bacterial isolates had MAR index of 0.3 and above.Conjugation studies revealed that nineteen out of twenty-six

donor isolates transferred resistant trait to the recipients while plasmid analysis yielded seven different plasmid profiles comprising one or two plasmids numbers with estimated sizes between 2.512kb and 10kb. This study showed that transfer of multi-antibiotic resistant gene to other pathogenic bacteria could result in serious health concern; therefore, packaged milk products industries should maintain high processing standards.

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND INFORMATION

Milk and milk products constitute important nutritional components for human diet and plays a prominent role in human nutrition (Javaid *et al*., 2009). Good quality milk meets the nutritional needs of the body better than any single food as it contains essential food constituents such as fat, proteins, carbohydrates, minerals, vitamins (Sharm and Joshi, 1992; Medhammar *et al*., 2012). As a result of the presence of these nutritional components, milk is an excellent culture medium for many microorganisms, especially bacterial pathogens (Henry and Newlander, 1997; Saeed *et al*., 2009). In order to extend the shelf life of milk for human consumption and prevent growth of spoilage organisms as well as prevent transmission of diseases via milk, this highly nutritious, versatile food is usually pasteurized (Edema and Akingbade, 2007). Unfortunately, many workers have reported post-pasteurization contamination of milk with resistant pathogenic bacteria (Brisabois *et al*., 1997; Oliver *et al*., 2005). For instance, some potential human pathogens, such as *Mycobacterium paratuberculosis*, *Bacillus cereus*, *Clostridium spp, Listeria monocytogenes* and *Salmonella spp* have been reported to survive conventional heat pasteurization in milk (Stabel *et al.*, 1997; Smith *et al.*, 2002; Torkar and Teger, 2008). Microbial contamination of milk has been reported to be responsible for deterioration of the quality of packaged milk (Frazier and Westhoff, 1986; Guerra *et al*., 2003).Approximately 50 % of the milk produced is consumed as fresh or pasteurised, one sixth as yoghurt or curd and the remaining utilized in the production of varieties of milk products such as ice cream and butter (Anjum *et al*., 1989; Lindmark*et al.,* 2003).

Pathogenic micro-organisms commonly isolated from contaminated milk have been reported to be resistant to antibiotics frequently prescribed in hospitals in Nigeria (Oladipo and Omo- Adua, 2011).

These pathogenic microbial contaminants in milk have been a major factor for public health concern since the early days of dairy industry (Altug and Bayrak, 2003).

Bacterial contamination can generally occur from three main sources; within the udder, outside the udder, and from the surface of equipment used for milk handling and storage (Oliver *et al*., 2005). Cow health, milking procedures, equipment sanitation and environment, such as water and personnel can influence the level of microbial contamination of raw milk (Farzana *et al*., 2009). Equally important is the milk holding temperature and length of time milk is stored before testing and processing that allow bacterial contaminants to multiply. These factors will influence the total bacterial count and the types of bacteria present in raw bulk tank milk. Another source of contamination by bacterial pathogen is unclean teats (Altug and Bayrak, 2003). The use of unclean milking and transporting equipment contributes to poor hygienic quality (Bonfoh *et al*., 2003).

In order to produce milk of good hygienic quality, it is therefore important to have clean healthy cows and clean utensils for milking and storage of the milk. Unfortunately, the consumption of unpasteurized milk in most developing countries including Nigeria has not attracted the desired attention.

Bacteria are widely distributed in nature and may be introduced into milk easily. Consequently, a broad spectrum of bacteria such as*Staphylococcus aureus, Escherichia coli,Salmonella spp, Pseudomonas spp, Enterobacter spp, Klebsiella spp, Proteus spp* and *Yersinia spp* have been recovered from rawmilk (Ayebo *et al*., 1976; De Buyser *et al*., 2001; Sivapalasingams *et al*., 2004) and some of these have been determined tobe potentially pathogenic and toxicogenic, and implicated in milkbornegastroenteritis (Bergdoll, 1979;Maguire *et al*., 1992). However, some of them including *Proteus spp* and *Klebsiellaspp* are rarely associated with foodborne infections. *Klebsiella pneumoniae*, the main Klebsiella pathogenic species causes pneumonia while *Proteusspp* has been reported to be mainly associated with wound and urinary tract infections. Thus the occurrence of these organisms in milk may pose risk to consumers.

Most of the other bacteria identified in milk have been implicated in milk and other food related infections (Kivaria *et al*., 2006).

Staphylococcus aureus by far is the most frequent pathogen associated with outbreaks of milk-borne infections (85.5% of the outbreaks), followed by *Salmonella* (10.1%) (De Buyser *et al.,* 2001). *Staphylococcus aureus* has been reported associated with food borne intoxication through production of enterotoxins, and may be introduced into milk from the udder or skin of humans. Other organisms in milk that have been reported belong to the enterobacteriaceae family. Enterobacteria organisms have been found to be common inhabitants of the intestinal tract of various domestic animals including cow, and are commonly found in cow dung which has been observed to be abundant at milking environments, and therefore easy contamination of the milk as a result of poor sanitation or milking environments (Kivaria *et al*., 2006).

Food products especially raw milk has been reported to be commonly contaminated with food borne pathogens and many of them show resistance to different antibiotics. Milk products are often contaminated with enterotoxigenic strains of *S. aureus* (Chao *et al*., 2007). It is currently not possible to effectively and consistently exclude such multiantibiotic resistant bacterial strains from the human food chain, which means that they continue to pose a significant clinical threat to consumers and concomitant economic threats to the food production and processing industry (Walsh *et al*., 2005). Presence of enterotoxigenic and antimicrobial resistant strains of bacterial pathogenshas become remarkably widespread in milk (Normanno *et al*., 2007). This requires a better control of food contamination sources and distribution of antimicrobial-resistant organisms (Normanno *et al*., 2007).

Food safety has raised public concerns, which may necessitate the actual sterilization of many milk products in future. Though sterile milk is now available, the heat required for sterilizing it, has been reported to alter its taste and marketability (Smith *et al.*, 2002).

1.2 STATEMENT OF RESEARCH PROBLEM

Foodborne diseases have a major public health impact and their well-publicized and widespread outbreaks have created an awareness of their potential threats to human health. The epidemiology of foodborne diseases is rapidly changing as newly recognized pathogens emerge and well-recognized pathogens increase in prevalence or become associated with new food vehicles (Alterkruse *et al.*, 1997).

There have been numerous outbreaks of milk-borne diseases in humans with pathogens such as *S. aureus*, *E. coli*, *Salmonella spp*, *Yersinia spp* and *Enterobacter spp* being implicated within this past Century, especially since mass production came into effect (Yagoub et al., 2005).

Milk can act as a vehicle for the transmission of bacterial diseases such as, salmonellosis, *E. coli* infections, cholera, brucellosis, streptococcal infections and listeriosis.

Infection with enteropathogenic *E. coli* usually, results in mild illness; however, some serotypes are enterohemorrhagic and can lead to hemolyticuremic syndrome. *Escherichia coli* 0157:H7 is the most common entero-hemorrhagic strain (Van Kessel *et al.,* 2003), which have been reported to cause acute kidney failure in children in the United States (Alterkruse *et al.*, 1997). Because *Salmonella* and *E. coli* 0157:H7 are shed in the animal's faeces, there is a risk of these pathogens entering the milk (Van Kessel *et al.*, 2003).Diarrhea disease has been a major public health problem causing high morbidity and mortality among children for many years (Bureau of Epidemiology, 2004). *Salmonella* causes diseases ranging from diarrhea to septicemia.

Milk contamination with antibiotic resistant bacteria can be a major threat to public health, as the antibiotic resistant determinants can be transferred to other pathogenic bacteria potentially compromising the treatment of severe bacterial infections. The prevalence of antimicrobial resistance among foodborne pathogens has increased in recent decades (Davis *et al.*, 1999; Garau *et al.*, 1999;

Threfall *et al*., 2000; Chui *et al*., 2002). In addition, the lack of stringent controls on antibiotic usage in human health and particularly in animal production systems increases the risk of antibiotic resistant milk borne pathogens.

As milk and milk products play an important role in human nutrition throughout the world, the products must be of high hygienic quality. In less developed areas and especially in hot tropics high quality of safe product is most important but not easily accomplished (DeGraaf *et al*., 1997). This is required since milk is also a suitable substrate for microbial growth and development. The fluid or semi-fluid nature of milk and its chemical composition (containing the essential nutrients) renders it one of the ideal culture media for microbial growth and multiplication (Ashenafi and Beyene, 1994; Teka, 1997). Mainly because of this reason, milk and milk products are more prone to the harbouring and proliferation of microorganisms.

In Nigeria, several workers have reported milk products to be contaminatedwith several bacterial pathogens such as*Staphylococcus spp*, *E. coli*, *Klebsiella spp*, *Enterobacter spp*and *Salmonella spp*(Umoh *et al*., 1990; Adeleke *et al*., 2000; Uzeh *et al*., 2006; Okpalugo *et al.,* 2008; Yabaya *et al*., 2012). The presence of these bacteria in milk products poses health hazards to consumers in this area. The presence of coliform organismsin milk has been linked to a wide variety of human infections such asendocarditis, urinary and genital tract infections, meningitis and septicemia (Mannu *et al*., 2003). Evidence indicate that *Salmonella* spp is one of the most etiologic agents responsible for several outbreaks associated with the consumption of milk (De Buyser *et al*., 2001)

1.3 JUSTIFICATION

Milk is considered an attractive source of energy, proteins and calcium for infants and young children who have few alternative sources for these nutrients. Besides its beneficial effects on nutrition, Milk

borne illnesses have been recognized since early days in the dairy industry (Ryser, 1998). Pathogenic bacteria in milk have been a major public health problem due to the number of diseases caused by them (Grant *et al*., 1995). In view of the health hazard associated with the consumption of contaminated milk, the findings from this study will help in evaluating the quality of milk within the study area in order to safeguard the health of the people.

Milk helps in fighting against diseases such as gout, kidney stones, breast cancer, rheumatoid arthritis, migraine headaches, amongst others. There are more than six billion consumers of milk and milk products, the majority of them in developing countries like Nigeria, as milk is a key contributor to improving nutrition and food security in these countries (Wesley, 2012). In Nigeria and other developing countries where surveillance and reporting of food-borne diseases are non-existent, it is extremely difficult to estimate how far milk products contribute to infection and diarrhoeal diseases (Ehiri *et al*., 2001). This makes it extremely necessary to undertake study on the quality of packaged milk sold in the area of study.

Milk may contain both pathogenic and nonpathogenic organisms. Pathogenic organisms such as species of *Staphylococcus, Escherchia*may come directly from the cow's udder. Various other pathogenic causing diseases like cholera and typhoid may find access in the milk from various other sources, which may include water, and the persons handling the milk. Nonpathogenic microflora may come directly from the udder and may also enter in the milk from milker's hands, utensils, cow barn, water, etc. (Hahn, 1996). These bacteria may cause undulant fever, dysentery, salmonellosis and tuberculosis (Feresu and Nyati, 1990)

Outbreaks of milk-borne diseases have occurred despite pasteurization caused either by improper pasteurization or recontamination thereby posing some risks to the consumers (DaSilva *et al.*, 1998). Milk products have been contaminated with pathogens that are resistant to several antibiotics. It is

currently not possible to effectively exclude such antibiotic resistant strains from milk products. This means that they continue to pose significant clinical threat to consumers and economic threats to the milk processing industry (Walsh *et al*., 2005).

Generally, food has been identified to be a very efficient vehicle for bringing a large number of people into contact with a potential hazard (Jordan, 2007). Thus, from a population perspective, food-borne exposure and milk in particular may be the most critical pathway for transfer of antibiotic resistant microbes to humans. Yet, data of antibiotic resistant microbes from milk is scanty and scattered. To be able to reduce considerably the problem of antibiotic resistance in the country, there is need to undertake a study on antibiotics susceptibility of bacteria isolates from milk products.

1.4 AIM OF THE STUDY

To isolate bacterial contaminants from packaged milk sold in Zaria, Nigeria, evaluate their susceptibility to commonly used antibiotics and examine resistance determinants.

1.5 OBJECTIVES

I .To isolate and identify bacterial contaminants found in packaged milk using pour plate method.

2. To determine the antibiotics susceptibility of bacteria isolated from packaged milk using disk diffusion method.

3. To determine minimum inhibitory concentration of selected antibiotics against resistant bacterial isolates using agar plate dilution method.

4. To determine whether resistant isolates are plasmid mediated or not using agarose gel electrophoresis.

5. To determine whether resistant factors are transferable by conjugation method.

6. To determine the sizes of plasmids isolated from the different resistant bacteria.

1.6 HYPOTHESIS

Packaged milk products marketed in Zaria, Nigeria are not contaminated with bacterial pathogens.

1.7 LIMITATIONS

Only aerobic bacterial contaminants were isolated. Anaerobics in milk products such as Lactic acid bacteria were not included in the study.

CHAPTER TWO

LITERATURE REVIEW

2.1 OVERVIEW OF MILK QUALITY

Raw or processed milk has been reported as a well-known good medium that supports the growth of several bacteria with resultant spoilage of the product or infections/intoxications in consumers (Murinda *et al*., 2004; Oliver *et al*., 2005). Microbes may gain entry into raw milk directly from dairy cows experiencing subclinical or clinical mastitis (Rodojcic-Prodaova and Necev, 1991), from the farm environment particularly the water source and utensils used for the storage of milk on farm or during transportation (Murphy and Boor, 2000). Microorganisms in milk have been observed to undergo rapid multiplication at high ambient temperatures (Jayarao and Henning, 2001; Gillespie *et al*., 2005; Hussein and Sakuma, 2005). A number of bacteria including *S*. *aureus*, *Escherichia coli* and *Salmonella spp* have been recovered from raw milk (De Buyser *et al*., 2001) and some of these havebeen determined to be pathogenic and toxigenic, and implicatedin milk-borne gastroenteritis (Maguire *et al*., 1992; De Buyser*et al*., 2001). In recent years,*E. coli* O157:H7 strain has been reported as a very important milk-borne pathogen and cattle has been implicated as its main reservoir (Betts, 2000). The antimicrobial resistance of *S. aureus* and *E. coli* recovered from raw milk and milk products have been reported (Umoh *et al*.,1990; Adesiyun *et al*.,1997a; Makovec and Ruegg, 2003; Pitkala *et al*., 2004).

Milk is highly valued and has been reported to provide essential nutrients in higher amounts than other staple foods (Oyawoye *et al*., 1997). Milk has been reported to be utilized in the production of at least 400 different fermented products all over the world (Prescott *et al*., 2008). Health complications associated with consumption of inadequately pasteurized milk products include serious infections with antibiotics therapeutic failure due to antibiotic resistance development. Antibiotics reportedly used to

treat infectious disease have been implicated in the development of multiple antibiotic resistances thereby rendering the antibiotic treatment ineffective (Johnston *et al.,* 1983; Devriese *et al.,* 1997). It has been estimated that nearly equal tonnage of antimicrobial agents are used in man and in agriculture worldwide (European Federation of Animal Health, 1997). When low doses of antibiotics are used, they inhibit the growth of susceptible bacteria while resistant bacteria thrive and grow such as in the presence of tetracycline (Eichner and Gravitz, 1999).

Pasteurization has been regarded as an effective method to eliminate bacterial pathogens in milk. However, the increasing number of reports on detection of bacterial pathogens in pasteurized fluid milk and ready-to-eat dairy products clearly indicates inherent failure of pasteurization methods of milk and milk products. Consumption of raw milk has been recognized as a major route of bacterial infection. There are several reports on consumption of raw milk, faulty pasteurized milk, or dairy products linked directly to human bacterial infections (Fashey *et al*., 1995; Evans *et al*., 1996).

2.2MILK COMPOSITION AND NUTRITIVE VALUE

Milk may be defined as the secretion of the female mammal used for the feeding of her young, and has been described as close to being nature's perfect food (Ensminger, 1993; Nickerson, 1999). The substances in milk have been reported to provide both energy and materials necessary for growth and maintenance of health. Bovine milk is commonly consumed by majority throughout the world, however, in some regions goat's milk or sheep milk may be more commonly used (Dogan *et al*., 2002). Fresh milk is neutral or slightly alkaline but on souring becomes acid because of the lactic acid formed by bacterial action on lactose. It has a water content of 88% and 12% of solids which constitute 3.5% fats, 0.6% salts, 4.8% sugars, and 3.1% protein (Stewart, 1978; Pape-Zambito *et al*., 2007). It has a wide range of positive nutritional benefits and supplies a variety of nutrients including protein for bodybuilding, vitamins, minerals (especially calcium), fat and carbohydrate for energy (Medhammar, 2012).

2.2.1 Milk Fat

Milk fat being an animal fat, is characterized as being saturated fat. However about 32% of milk's fatty acids are unsaturated, primarily as mono-unsaturated acids like oleic acid (C18:1). Milk supplies the essential fatty acids linoleic acid (2.1%), lanoleic (0.5%) and arachidonic acid (0.14%). These are required by the human body for normal metabolism and growth. Short (C2 to C6) and medium chain (C8 to C12) fatty acids account for about 12% of the fatty acids of milk and being more readily digested. They do not contribute to the elevation of blood lipids nor are they deposited in adipose tissue (Lee and Gerrior, 2002).

2.2.2 Proteins

Proteins are valuable component of milk in terms of their importance in human nutrition and their influence on the properties of dairy products containing them. Proteins are the body's 'building blocks' affecting growth and immunity. Antibodies, enzymes and hormones all contain proteins, thus the proteins we eat provide the amino acids needed to replace both these and essential body cells. Whilst the body is able to synthesise some amino acids, there are eight essential amino acids such as isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine which have to be supplied in the diet (Furst and Stehle, 2004).

In milk, proteins can be classified into two, casein and whey proteins. Casein is recognized as the micellar framework which comprises a network of alpha casein complex with calcium phosphate. It is the most commonly used milk protein in the food industry and contains 21 amino acids. Acid casein, a granular milk protein, is available in two types - edible and technical. Edible acid casein is highly

nutritional, low in fat and cholesterol, and flavorful making it ideal for medical and nutritional applications (Fox, 1995).

When casein is removed from skim milk using precipitation method, the protein remains in the liquid solution and is called whey proteins or milk-serum proteins. It accounts for only about 20% of the total protein found in milk, while casein makes up about 80% of milk protein. Whey proteins are now well known for their high nutritional value and versatile functional properties in food products (De Wit, 1998; Harold, 2004). Milk protein has a very high nutritional value and is a rich source of essential amino acids (Harding, 1995).

2.2.3 Carbohydrate

The major carbohydrate in milk of most mammals is lactose, usually called milk sugar. It is water soluble occurring as a soluble molecule in milk. Lactose with the exception of water is, at about 4.6%, the principal component of milk; however, it is the less important of the solids both nutritionally and commercially. Lactose consists of two molecules, D-glucose and D-galactose and is digested or broken down into these constituents by the enzyme lactase (Mustapha *et al*., 1997). Lactose is a useful source of dietary energy. However, adults in certain racial groups lose the ability to digest it and suffer discomfort and other symptoms of digestive upset as a result of consuming substantial quantities of dairy products containing lactose, a condition called lactose intolerance (Vesa *et al*., 2000).

2.2.4 Minerals

Many trace elements essential for health and growth, are present in milk. Sodium, calcium, potassium and phosphorus account for about 4% by weight of the fat-free human body. Some of the trace minerals are, zinc, cobalt, iodine, iron, etc (Stewart, 1978; Gaucheron, 2005). Minerals in milk provide constancy of osmotic pressure. This property can prevent the depression of freezing point temperature. The

amount of minerals in milk can provide the recommended daily allowance for calcium and 75% for phosphorus (Renner *et al*., 1989). These minerals are widely recognized as important factor for bone development and growth of children (Okolo *et al*., 2000).

2.2.5 Vitamins

Vitamins are complex organic substances that are needed in very small amounts for many of the processes carried out in the body. Usually only a few milligrams (mg) or micrograms (µg) are needed per day, but these amounts are essential for health. Most vitamins cannot be produced within the body, and as a result needs to be provided in the diet, although vitamin D can be obtained by the action of sunlight on the skin, and small amounts of a B vitamin (niacin) can be made from the amino acid (tryptophan). Milk is a source of 12 water-soluble vitamins and four fat-soluble vitamins (Harding, 1995).

Vitamins in milk are readily affected by processing. Some of them are heat-sensitive. Storage conditions also affect vitamins, exposure to light or oxygen can cause loss of some vitamins (Varnam and Sutherland, 1994). Both storage and processing condition must be considered to prevent loss of vitamins in the products.

2.3SOURCES OF BACTERIAL CONTAMINATION IN MILK PRODUCTS

The isolation of pathogenic and coliform bacteria from milk indicates that milk may be contaminated from udder of animals, utensils used for milking or the water used as well as handlers (Bonfoh *et al.,* 2003). The quality of the starting raw milk has a very definite effect on the yield and quality of products made from it. The compositional quality, the hygienic quality, the health of the cow and the level of contaminants present can all have an impact on the yield and quality, and hence financial return from products made from milk (Harding, 1995). Inadequate cooling of the milk, improper udder preparation methods, unclean milking equipment and the water used for cleaning purposes are considered as the

main source of milk contamination (DeGraaf *et al*., 1997). In order to produce milk of good bacteriological quality, there is need to be aware of the sources of contamination and importance of proper milk handling, cooling and storage.

2.3.1 Contamination from Cow's Udder

Raw milk as it leaves the udder of healthy cows normally contains very low numbers of microorganisms and generally will contain less than 1000 total bacteria per ml (Murphy, 1996; Godefay and Molla, 2000). Natural flora originating from the cow generally has little influence on total aerobic plate counts (Murphy and Boor, 2000). The bacterial infection of milk taking place inside the udder is called primary infection. The main groups of microorganisms for this infection are the aerobic mesophilic microflora, and they contribute little to the deterioration of good quality raw milk (<5000 cfu/ml) (International Dairy Federation, 1996).

In case of mastitis counts of *Streptococci*, *Enterococcus*, *Staphylococci* or coliforms will be as high as the total aerobic plate count and can be very high up to 10^7 cfu/ml under certain circumstances (Slaghuis, 1996). Cow with mastitis has the potential to shed large numbers of microorganisms into the milk supply (Bramley and McKinnon, 1990). Detection of implied pathogens does not necessarily indicate that they originated from cows with mastitis. Potential environmental mastitis pathogens and similar organisms can occur in milk as a result of other contributing factors such as dirty cows, poor equipment cleaning and poor cooling.

The exterior of the udder can be an important source of contamination. But the exterior of the udder is influenced by the environment of the cows, in which cows are housed and milked (Murphy, 1996). In temperate regions, cows are housed in winter and pastured in summer. Differences in teat contamination can be found between housing and pasturing (International Dairy Federation, 1994a).

Both total aerobic plate and aerobic spore counts are lower when cows are at pasture. When cows are housed, bedding material and feedstuffs can be contamination sources. In either cases (housing and pasturing) feaces or dung is also an important contamination source. Teat surfaces are also a source of bacterial spores in milk. Pathogenic bacteria that might contaminate the teats are *Salmonella typhii, Salmonella dublin*and *Yersiniaenterocolitica*. Faecal contamination is very likely to occur (International Dairy Federation, 1996). Damaged teats can affect milk quality in that any break in the skin can become a reservoir for mastitic bacteria and give rise to a significant increase in bacterial count.

2.3.2 Contamination from Environment

The environment around the farm is a good source of contamination. The milking place, dirt and even air can be sources of microbes in milk. Contamination of environment varies from place and season. However, the contaminated microbes should be pathogens such as *Salmonella spp* or *Campylobacter spp* (Phillips and Griffiths, 1990). Milking area should be cleaned before milking commence. Most of the dirt and soil should be removed. The milk reservoir should be in close vessel to prevent contamination from dust. Furthermore, milk should be filtered with sieve or fabric before pouring into storage tank for further processing.

2.3.3 Contamination from Milking Equipment and Storage

Bacteria are present in the air, dust and water, especially any water containing traces of milk residues which may have been left in the milking plant overnight, as such residues provide a very good source of food for bacteria, thereby enabling the bacterial counts to increase rapidly. Cleaning regimes are based on removing visible dirt, removing milk residues (fat, protein, milkstones) which harbour bacteria, then sterilization of the cleaned surfaces using heat or chemical sterilants such as sodium hypochlorite (Harding, 1995). Cleaning and sanitizing procedures can influence the degree and type of bacterial

growth on milk contact surfaces by leaving behind milk residues that support growth, as well as by setting up conditions that might select for specific bacterial groups. More resistant bacteria may endure in low numbers on equipment surfaces that are considered to be efficiently cleaned with hot water.

The influence of cleaning and disinfection on the survival of bacteria on milk contact surfaces is not yet fully understood. Attachment of bacteria to different surfaces (Husmark and Ronner, 1990) and possible scaling may cause problems with cleaning and disinfection. In most cases not all bacteria are killed and removed during cleaning and disinfection.

The multiplication of bacteria in milk has been observed to be dependent on the temperature and time of storage. After production, milk can be stored in cans and in bulk tanks before collection. The storage temperature influences the types of bacteria which grow and their spoilage characteristics. Spoilage of raw milk has been reported to be due to coliforms, resulting in souring of milk. During storage in bulk tanks and transport, the microflora of the milk changes from micrococci to psychrotrophic gram-negative rods. There are many different microorganisms (mainly bacteria), which can find access to milk, and there are three broad temperature ranges classifying their optimum growth rates. Organisms with an optimum growth rate at low temperatures (0-15°C) are psychrophiles, example, *Pseudomonas spp*, at medium temperatures (20-40°C) are called the mesophiles, example, *Salmonella spp* and at high temperatures (45- 55°C) the thermophiles, example *Bacillus spp* (International Dairy Federation, 1996).

2.4BACTERIOLOGICAL QUALITY OF MILK

Milk has been considered as one of the most important primary foods, however, several bacterial pathogens have been detected in milk including enterohaemorragic *Escherichia coli*, *Staphylococcus aureus, Salmonella specie*, and *Yersinia enterocolitica*(Pazakova *et al*., 1997; Canganella *et al*., 1998; Dineen *et al*., 1998;Reed and Grivetti, 2000; Proctor and Davis, 2000; Gulmez and Guven, 2003;CDC,

2003; Mazurek *et al*., 2004; Tekinşen and Özdemir, 2006). Bacterial pathogens from milk also include psychrotrophic microorganisms, mainly belonging to the genus *Pseudomonas*, that are responsible for the spoilage of milk and dairy products owing to their ability to produce heat-resistant proteolytic and lipolytic enzymes at chill temperatures (Gilmour and Rowe, 1990). Their enzymes can withstand heat treatments of pasteurization and ultrahigh temperature treatments (UHT) (Lopez-Fandino *et al*., 1993; Koka and Weimer, 2001). These pathogens have been linked to livestock, feed, and storage environment (Marco and Wells-Bennik, 2008). The bacteriological quality of milk is strictly related to the management practice, such as equipment and environmental hygiene, cow wellness, packaging and handling (Little *et al*., 2008).

The incidence of milk-borne infections has markedly increased over the last 20 years, with nearly a quarter of the population at higher risk for illness today (Oliver *et al*., 2005). Milkborne disease surveillance began in the US in the early 1900s in response to morbidity caused by milk-transmitted typhoid fever and infantile diarrhoea (Cliver, 1990). From 1998 to 2005, the Centers for Disease Control and Prevention (CDC) identified 45 outbreaks of milk-borne illnesses that involved unpasteurized milk, or cheese made from unpasteurized milk, accounting for 1.007 illnesses, 104 hospitalizations, and two deaths (CDC, 2003).

Many of these milkborne diseases that historically caused significant mortality and morbidity were largely eradicated in the industrialized world as a result of sanitation and pasteurization, disease control efforts in animals and other measures (Tauxe, 2002). Although many milkborne infections are controlled, the burden of emerging bacterial pathogens remains substantial.

2.4.1 ***Escherichia coli***

Escherichia coli a common inhabitant of the human and animal intestinal tract is a Gram- negative, facultative aerobic organism, and a member of enterobacteriaceae family (Nys *et al*., 2004; Von Baum and Marre, 2005). Organisms of this species are generally lactose fermentors, but sometimes the lactose fermentation is delayed (Cliver, 1990). Most strains of *E. coli* are harmless; however some are pathogenic causing severe intestinal and diarrhoeal diseases (Meng *et al*., 2001, Kaper *et al*., 2004). These potentially harmful *E. coli* are classified into categories based on the production of virulence factors and on the clinical manifestations that they cause. They havebeen reported in raw milk and milk products by several authors (Aly and Galal, 2002; Soomro *et al*., 2002; Lues *et al*., 2003; Chye *et al*., 2004). In addition to the presence of *E. coli* denoting fecal pollution, the presence of virulence – related genes in *E. coli* strains refer to the pathogenicity of the isolates. Previous studies documented the aquation of some *E. coli* isolates from raw milk and milk products for virulence markers (Klie *et al*., 1997; Jayarao and Henning, 2001; Holko *et al*., 2006; Paneto *et al*., 2007).

Pathogenic *E. coli* falls into two groups: the first one is the urogenic group, which is the predominant causative organism of urinary tract infections (UTI), is also frequently isolated in neonate meningitis and Gram-negative nosocomial and community-acquired infections. The other is the enteric group that often causes childhood enteritis and bacteria-related traveller's diarrhea (Von Baum and Marre, 2005). Among the enteric *E. coli*, Shiga-toxin (Stx) producing *E. coli* (STEC) O157:H7 and non- O157:H7 have been identified as aetiological agents for haemorrhagic uremic syndrome (HUS) in humans (Von Baum and Marre, 2005). However, of the two, O157:H7 serotype is considered as being the most significant and has been associated with large food-borne outbreaks in North America, Europe, and Japan (White *et al*., 2002). The Centre for Disease Control (USA) estimates that *E. coli* O157:H7 causes approximately 73,000 illnesses and 61 deaths each year in the USA while non-O157:H7 STEC account for an additional 37,740 cases with 30 deaths (White *et al*., 2002). In humans, these infections are associated with gastroenteritis

that may be complicated by hemorrhagic colitis (HC) or hemolytic-uremic syndrome (HUS), which is a major cause of renal failure in children (Mora *et al*., 2004).

Generally antibacterials are not recommended for therapy of STEC infections because they can lyse cell walls therefore releasing the toxins (Waterspiel *et al*., 1992; Wong *et al*., 2000). Additionally, they are usually avoided because they can also cause increased expression of the toxins *in vivo* (Zhang *et al*., 2000). Despite the general practice of not using antibacterials to treat STEC infections, there have been recent reports suggesting that antiobiotic multi-resistance of STEC is on the rise (Galland *et al*., 2001; Willshaw *et al*., 2001; Schroeder *et al*., 2002,).

2.4.2 *Salmonella species*

Salmonella species includes more than 2500 different serotypes and represents a leading cause of milkborne infections worldwide (White *et al*., 2002; Chen *et al*., 2004; Magistrali *et al*., 2008). Nearly 1.4 million cases of salmonellosis occur each year in the United States, of which 95% are foodborne cases (Mead *et al*., 1999). *Salmonella* species cause a wide range of human disease such as enteric fever, gastroenteritis, Bacteremia (Bennasar *et al*., 2000). A variety of foods have been implicated as vehicles transmitting salmonellosis to humans, including raw meats, poultry, milk and dairy products (Forsythe, 2000) as well as fish, shellfish, fresh fruits and juice, and vegetables (Gomez *et al*., 1997). Contamination has been reported to be through poor temperature control and handling practices, or cross-contamination of processed foods from raw ingredients. The primary reservoir is the intestinal tract of humans and animals. This pathogen is excreted in the faeces and can remain viable in the faecal material for several years. The principal source of Salmonella infection has been reported to be ingestion of contaminated food. Consumption of contaminated milk may lead to a number of gastrointestinal bacterial infections. Gastroenteritis has been attributed to species of *Salmonella* especially *Salmonella typhimurium* and *Salmonella enteridis* and symptoms occur 7-72 hours following

ingestion of contaminated food. Typhoid and paratyphoid fevers are caused by organisms such as *Salmonella typhi* and occur less frequently than outbreaks of gastroenteritis (International Dairy Federation, 1994b).

Currently the increasing prevalence of multidrug resistance among *Salmonella* and resistance to the clinically important antimicrobial agents such as fluoroquinolones and third-generation of cephalosporins has also been an emerging problem in China and other countries (Brands *et al*., 2005; Gebreyes and Thakur, 2005; Chao *et al*., 2007). Typhoid fever is a global problem with an estimated 12-33 million people occurring in worldwide (Kohinur *et al*., 2010). Fluoroquinolones are the drugs of choice for treating human salmonellae infections, while other antimicrobials are not clinically effective and contribute to a prolonged carrier status (Anderson *et al*., 2003; Skov *et al*., 2007). However, there are increasing reports describing decreasing susceptibilities to antimicrobial agents such as fluoroquinolones and expanded spectrum cephalosporins, drugs of choice in cases of life threatening salmonellosis due to multidrug-resistant strains (Threlfall, 2002; White *et al*., 2002). A recent study in Spain revealed that ampicillin resistance in *Salmonella* species had increased from 8% to 44%, tetracycline resistance from 1% to 42%, chloramphenicol resistance from 1.7% to 26%, and nalidixic acid resistance from 0.1% to 11% (White *et al*., 2002). In the USA, resistance to tetracycline in *Salmonella* species increased from 9% in 1980 to 24% and ampicillin resistance increased from 10% to 14% (White *et al*., 2002). A recent survey in Portugal revealed that only 25% of the Salmonella isolates obtained were susceptible to all antimicrobials, 39% were resistant to one antimicrobial and 36% were resistant to two or more agents of different groups (Antunes *et al*., 2002). In the Indian subcontinent and South East Asia, it is a norm for *S. typhi* strains to exhibit multidrug resistance (Threlfall, 2002).

2.4.3 *Enterobacter species*

Enterobacter speciesbelongs to the Family *Enterobacteriaceae* that contains a number of species including *E. agglomerans*, *E. cloacae*, *E. sakazakii*, *E. asburiae,E. aerogenes* and *E. liquefaciens*. The differentiation among these species is based on biochemical reactions, serological and molecular techniques (Hoffmann and Roggenkamp, 2003; Iversen *et al*., 2004b). They are frequently isolated from clinical samples and food products and are considered opportunistic pathogenthat have been implicated in severe forms of necrotizing colitis(Van Acker *et al*., 2001) and meningitis (Bar-Oz *et al*., 2001) especially in neonates witha mortality rate varying from 40% to 80%. *Enterobacter* species have beenreported as frequently isolated from different environmentsincluding soil, milk powder factories, chocolatefactories and households (Kandhai *et al*., 2004). Theyhave been also isolated from a wide range of foods including ultra high-temperature treated milk (UHT milk), cheese, meat, vegetables, grains, sorghum seeds, rice seeds, herbs, spices, fermented bread,fermented beverage, tofu, and sour tea (Gassem, 1999, 2002; Leclercq *et al*., 2002; Iversen and Forsythe, 2003, 2004a),

Enterobacter species are responsible for variety of infections (Sinave, 2003). These infections include bacteremia, lower respiratory tract infections, skin and soft tissue infections, urinary tract infections, intra-abdominal infections, septic arthritis, osteomyelitis and ophthalmic infections.

Most of the enterobacter bacteria are innately resistant to older antimicrobial agents such as the beta-lactams and have the ability to rapidly develop resistance to newer agents like the quinolones. Resistant strains of Enterobacterto major groups of antimicrobial agents vary widely among published reports. For example, aminoglycosides, the percentage of strains resistant to gentamicin ranges from 0 to 51%. For ciprofloxacin, resistance varies from 0 to 36% of strains tested, and for trimethoprim-sufamethoxazole, resistance varies from 0 to 60% of strains (Thomson *et al*., 1994; Scriver and Low, 1995). These wide ranges suggest that numerous factors impact the occurrence of antimicrobial resistance among strains of *Enterobacter* spp such as increased use of the respective drugs in a given environment (Jones, 1994).

2.4.4 *Staphylococcus aureus*

These are Gram-positive, facultatively anaerobic, non-sporeforming cocci. They were described in 1897 (Forsythe, 2000). This pathogen produces a wide range of pathogenicity and virulence factors like staphylokinase, hyaluronidases, coagulases and haemolysins (Forsythe, 2000). Staphylococcal food poisoning has been reported to be caused by the ingestion of food containing pre-formed toxins, named enterotoxins secreted by *S. aureus*. It is considered one of the leading food-borne illnesses in human worldwide and is associated with contaminated food of animal origin such as milk and dairy products, meat and meat products (Tsegmed *et al.,* 2007).

The type of food poisoning caused by *S. aureus* is characterized by nausea, characteristic projectile vomiting, and abdominal cramps, often with diarrhoea but without fever. The onset of the symptoms is rapid, often appearing 1-6 h after ingestion of the contaminated food depending on individual susceptibility and toxic dose ingested. (Le Loir *et al*., 2003).

Staphylococcus aureus has been a major causative agent of mastitis which is the most economically important diseases for the dairy industry so more effective therapeutic treatment and prophylactic approaches are surely needed (Chiang *et al*., 2007; Oviedo-Boyso *etal*., 2008). *Staphylococcus aureus* can gain access to milk either by direct excretion from udders with clinical and subclinical staphylococcal mastitis or by environmental contamination during the handling and processing of raw milk (Scherrer *et al*., 2004; Jørgensen *et al*., 2005). Staphylococcal enterotoxins (SEs) are serologically grouped into five major classical types which are staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), staphylococcal enterotoxin C (SEC), staphylococcal enterotoxin D (SED) and staphylococcal enterotoxin E (SEE) in addition to toxic shock syndrome toxin (TSST-1) which causes toxic shock syndrome in human, SEA and SEB are usually more common in milk and milk products (Chiang *et al.,* 2006).

The resistance to antimicrobial agents among staphylococci is an increasing problem; these strains have been reported to frequently show multiple antimicrobial resistance patterns particularly to methicillin and vancomycin (Enright, 2003;Reinoso *et al*., 2006; Normanno, *et al*., 2007;Dizbay *et al.,* 2008).The administration of antibiotics to food-producing animals, for therapeutic purposes or as growth promoters, could be a primary selection factor for antimicrobial-resistant bacteria pathogens. Increased attention has been focused on plasmid-encoded resistance to antiseptics and disinfectants in antibiotic resistant staphylococci (Bjorland *et al.,* 2003). Lindsay (2010) recorded that plasmids in *S. aureus* are predominantly of two types, small rolling circle plasmids often encode only one or two resistance genes, such as pT181 (Khan, 2005). The larger plasmids replicate by the theta mechanism and can carry a combination of resistance genes including penicillinase, heavy metals, detergents, trimethoprim and aminoglycosides, some of which are due to integrated small plasmids or transposons (Berg *et al.,* 1998). Some larger plasmids also encode the *tra* genes for conjugative transfer and many strains of *S. aureus* carry one or more plasmids (Lindsay, 2010). *Staphylococcus aureus* is one of the most common agents in bacterial food poisoning outbreaks. Its strains produce a spectrum of protein toxins and virulence factors thought to contribute to the pathogenicity of this organism (Adwan *et al.*, 2005).

2.4.5 *Pseudomonas species*

Pschrotrophic bacteria have been recognised as a recurring problem in refrigerated storage and distribution of perishable food products. The predominant bacteria limiting the shelf life of processed fluid milk has been reported to be *Pseudomonas* species (Dogan and Boor, 2003; Gunasekera *et al*., 2003). *Pseudomonas* species have been implicated in the spoilage of processed milk (Rajmohan *et al*., 2002). These species are able to grow to high numbers during refrigerated storage, and also produce heat-stable extracellular lipases, proteases, and lecithinases which can further contribute to milk spoilage (Ternström *et al*., 1993). Many of these enzymes remain active, even following thermal

processing. Even though they are easily inactivated through pasteurization or UHT-treatment, their heat resistant enzymes persist upon processing of the milk (Chen *et al*., 2003).

Degradation of milk components through various enzymatic activities can reduce the shelf life of processed milk. Lipases degrade the milk fat causing rancid, soapy and occasional bitter off-flavours through the formation of medium chain fatty acids. Proteases that degrade casein cause a grey colour, bitter off-flavours and gelation of UHT products (Datta and Deeth, 2001).

Pseudomonas species has been recognized as a potential opportunistic human pathogen and constitutes potential hazards to both human and animal health. It has been implicated in many types of infections and food poisoning outbreaks (Jay, 2000)

Pseudomonas species has been found to be resistant to aminoglycosides and quinolones (Alatossava and Alatossava, 2007).Its general resistance is due to a combination of factors such as low permeability of its cell wall, genetic capacity to express a wide repertoire of resistance mechanisms. It can become resistant through mutation in chromosomal genes which regulate resistance genes as well as acquire additional resistance genes from other organisms via plasmids, transposons and bacteriophages (Pitt and Sparrow, 2001). The possession of efflux pump systems capable of conferring resistance to a wide range of unrelated classes of antimicrobial agents has also been demonstrated in *Pseudomonas* species (Lomovskaya *et al*., 2001).

2.4.6 *Yersinia species*

Yersinia speciesare Gram negative, facultative anaerobic rod bacteria. The natural reservoir of infection has been reported to be the intestinal tract of wild and domestic animals (Quinn *et al*., 1999b). Although contaminated milk has been said to be a source of human infection due to *Y. enterocolitica*, animal reservoir and contaminated environment are also considered as other possible infection sources for

human (Schlundt, 2002). *Yersinia enterocolitica* and *Y. pseudotuberculosis* may contaminate raw milk and even pasteurized milk products thereby posing a public health risk.*Yersinia enterocolitica* has been isolated from raw milk in many countries, like Australia, Canada, Czechoslovakia, and USA. There were also a few reports on the isolation of this pathogenic strain associated with human disease from pasteurized milk (Ackers *et al*., 2000). This has been reported to be due to the malfunction in the pasteurization process leading to inadequate treatment or postprocess contamination, or it may be due to the contamination with heat-resistant strains of *Y. enterocolitica* (Bottone, 1999).

Yersinia enterocolitica has been reported to produce a heat-stable enterotoxin that has been associated with food-poisoning strains in humans and mesenteric lymphadenitis ("pseudo-appendicitis"); while *Y. pseudotuberculosis* was reported to cause mesenteric lymphadenitis, in addition to ilitis and septicaemia (Greenwood and Hooper, 1990). Clinical diseases in humans from both species of *Yersinia* are mainly observed in children and young adults. In New Zealand, 487 cases of yersiniosis were notified in 2006 (Ackers *et al.,* 2000). Surveys of Bulk Tank Milk in US states found 1.2% (Jayarao and Wolfgang, 2003) and 6.1% (Jayarao and Henning, 2001) of samples positive for *Y. enterocolitica.* Irish and French studies reported prevalence's of contamination of 39% and 36%, respectively (Rea *et al.,* 1992; Desmasures *et al.*, 1997). Rare cases of mastitis have been associated with *Y. pseudotuberculosis* in Israel *(*Shwimmer *et al.,* 2007). Reports from other workers have shown that risk factors for contamination of raw milk has been those associated with poor hygiene at milking and faecal contamination of the teat ends prior to milking cup attachment *(*Shwimmer *et al.,* 2007). The psychrotrophic nature of *Yersinia specie* has been reported to be of particular significance in milk and milk products that are normally stored at low temperatures. They can survive in the presence of high numbers of competing microorganisms and could maintain the virulence plasmid during extended storage at refrigeration temperatures (Larkin *et al*., 1991). In pasteurized milk, contamination has been mainly attributed to inadequate pasteurization or post process contamination (Kushal and Anand, 1999).

Systemic and extraintestinal infections and enterocolitis in immune-compromised patients require antibiotic therapy, and the agents used most commonly include chloramphenicol, gentamicin, tetracycline, cotrimoxazole and ciprofloxacin (Butler, 1990). Although there is data concerning the incidence of *Y. enterocolitica* and related species in foods in some countries (Siriken, 2002; Fredriksson-Ahomaa and Hannu, 2003; Soltan-Dallal and Moezardalan, 2004), but compared with other bacterial enteropathogenes, there are a few studies about the antimicrobial studies of *Yersinia* spp, which are isolated from milk products and human. Resistance to ampicillin and many cephalosporins is frequently observed among *Pseudomonas* species (Stock and Wiedemann, 1999; Tzelepi *et al*., 1999; White *et al*., 2002; Stock and Wiedemann, 2003). The expression of ß-lactamase enzymes A and B has already been associated with *Y. enterocolitica*, *Yersinia intermedia* and *Yersinia frederiksenii* (Stock *et al*., 1999, 2000; Stock and Wiedemann, 1999, 2003).

2.5BACTERIOLOGICAL QUALITY TESTS FOR MILK

Sanitary methods of handling milk must be strictly adhered to rigidly in order to provide safe milk for human consumption. Furthermore, since milk is a good growth medium, even a small number of non pathogens can multiply considerably if the milk is not kept refrigerated. A number of standard tests are carried out periodically on milk since consumers cannot determine milk contamination during purchase. From the results of these tests, milk is classified into grades designated as A, B, and C (Volk and Wheeler, 1980). Tests commonly employed to determine the quality of milk include dye-reduction (Methylene blue reduction and resazurine reduction), Alcohol test, Standard plate count, Coliform count, Somatic cell count, Titrable acidity, and phosphatase tests (Marshall, 1992).

2.5.1 Dye-reduction Tests

Dye- reduction tests have been employed to check for the overall microbial load and quality control of milk and other liquid foods (Impert *et al.,* 2002). These tests have been successfully employed to quantify viable cell count in milk within a very short time. The tests are less precise criterion for classifying milk according to its bacteriological quality. This calls for the need to periodically verify the quality of milk with more precise microbiological tests such as standard plate count (Ombui *et al.*, 1995).

2.5.1.1Methylene blue reduction test

Methylene blue is a blue-colored reagent used to estimate the bacterial population of a given milk sample (Nandy *et al*., 2007). A known dilution of the methylene blue solution is added to the milk sample and observation is made at fixed intervals until the blue color disappears. The number and species of organisms present in the milk determines the time required for the disappearance of the blue color in the milk (May *et al*., 2003; Nandy *et al*., 2007). Normally if the number of bacteria increases, the time required to decolorize the blue color is shorter. This test is usually used for grading the quality of raw milk before pasteurization. On the basis of this test, raw milk is graded as follows (Kurwijilla *et al*., 1992):

- Very good: not decolorizing in 5 hours.
- Good: decolorized in less than 4 hours, but not less than 3 hours.
- Fair: decolorized in less than in 2 hours, but not less than 1 hour.
- Poor: decolorized in less than ½ hour.

2.5.1.2Resazurin reduction test

This test is also used for grading the sanitary quality of milk by applying the chemical reagent resazurin. The procedure is similar to that of the methylene blue test, except that this test is quicker and the result is obtained in much less time (Reddy and Bordekar, 1999). Resazurin imparts blue color to milk which when reduced to resorufin changes to pink and finally to white on reduction to dihydroresorufin. The time required for complete decolorization, reduction of the resazurin and the degree of colour change is directly related to the number of bacterial organisms in the milk (Ombui *et al*., 1995; Teka, 1997). A comparator disc reading value of 4 and above at10 minutes with resazurin test indicates good quality while a comparator disc reading value of less than 4 at 10 minutes indicates poor quality milk (Ombui *et al*., 1995).

2.5.2 Alcohol Test

When milk contains more than 0.21% acid, or when calcium or magnesium compounds are present in greater than normal amounts, it coagulates on the addition of alcohol. This fact is the basis of alcohol test, which furnishes a means of judging the quality of milk (Ombui *et al*., 1995).

2.5.3 Standard Plate Count (SPC)

The standard plate count of milk gives an indication of the total number of aerobic bacteria present in the milk at the time of pick up. Obviously, very clean milk will have lower bacterial counts than milk collected or handled under unsanitary conditions. The standard plate count has been reported to be a good basis for grading the quality of milk (Volk and Wheeler, 1980). Milk samples are plated on standard plate count agar media and then incubated for 48 hrs at 32°C to encourage bacterial growth. Single bacterium or clusters of bacteria visible colonies are then counted. All plate counts are expressed as the number of colony forming units (CFU) per milliliter (Murphy, 1996). This method has been used to

estimate the bacterial population in milk. This method has a limited value in that it doesn't indicate the quality of microbial populations in terms of pathogens and non pathogens (Teka, 1997). The standard plate count is generally accepted as the most accurate and informative method of testing bacteriological quality of milk (Kurwijilla *et al*., 1992; Godefay and Molla 2000). It is sensitive but also labour intensive and is inaccurate for bacteria high count in milks (Slaughuis, 1996). Plate count standards have been developed to ensure satisfactory production hygiene and that the product is safe (Table 2). The plate count method has been conducted as a valuable adjunct to guide sanitarians in correcting sanitation failures and improving milk quality (International Dairy Federation, 1990).

Table 2.1: Grade of Milk Based on Standard Aerobic Bacteria Plate Count

Bacterial Count/ml	Grade
Not exceeding 200,000	Very Good
200,000 – 1,000,000	Good
1,000,000 – 5,000,000	Fair
>5,000,000	Poor

Source: Kurwijilla *et al.* (1992)

2.5.4 Coliform Bacteria in Milk

Coliforms are group of bacteria, which inhabit the intestinal tracts of human and animals. They are excreted in large number with human excreta and animal droppings. They may be found in the soil, on vegetables and in untreated water (Teka, 1997). It includes all aerobic and facultatively anaerobic, Gram-negative, non-spore forming rods able to ferment lactose with the production of acid and gas at 35°C within 48 hours. Most of them belong to the genera *Escherichia, Enterobacter* and *Klebsiella*

(Godefay and Molla, 2000). The presence of coliform organisms in milk indicates unsanitary conditions of production, processing or storage. Hence their presence in large number in dairy products is an indication that the products are potentially hazardous to the consumers' health (Volk and Wheeler, 1980; Godefay and Molla, 2000). Coliform organisms contaminate milk from unclean milker's hands, improperly cleaned and un sanitized or faulty sterilization of raw milk utensils especially churns, milking machines, improper preparation of the cows' flecks or dirt, manure, hair dropping into milk during milking, udder washed with unclean water, dirty towels and udder not dried before milking (Ombui *et al.,* 1995).

2.5.5 Tests for Specific Pathogens

Unless there is some evidence that a particular disease is being transmitted through milk, tests for specific pathogens are not run. The procedure to be followed depends on the specific organism in question (Quinn*et al.,* 1999a).

2.5.6 Somatic Cell Counts (SCC)

Somatic cell count refers to the total cells per millitre in milk. The somatic cell count (SCC) has been internationally recognized as a parameter for assessing milk quality and udder health (Degraaf *et al.*, 1997). European Union (EU) standards require that the milk should not contain more than 400,000 somatic cells/ml. Milk markets routinely rely on somatic cell counts to ensure a quality product. Somatic cell counts levels are monitored to ensure compliance with set milk quality standards. Today, most markets in developed countries pay a premium for low SCC, good quality milk. One can appreciate the reasons, for paying a bonus for quality milk when the relationship between mastitis (high SCC) and milk composition is understood. Chemical changes in milk composition due to mastitis reduce milk quality (Rice and Bodman, 1997).

2.5.7 Titrable Acidity Test

In order to determine the sourness of milk, titration is often used with sodium hydroxide (NaOH) and the degree of sourness is given by Soxhlet-Henkel Degree (°SH). Generally the sourness of normal milk is 6 to 7°SH. If the milk sourness is 4 to 5°SH, it indicates that either the milk is adulterated or there is mastitis (Kurwijila *et al.,* 1992).

2.5.8 Phosphatase Test

The phosphatase test is the most important public health measure for controlling the efficiency of pasteurization, hence the safety of milk. Phosphatase is an enzyme, which is normally present in raw milk. When milk is pasteurized by any of the recognized processes, the enzyme is completely inactivated. Therefore, a positive phosphatase test will indicate that the milk is not properly pasteurized. It may mean any one of the following (Teka, 1997):

- The pasteurization temperature time combination was not strictly observed
- The pasteurization equipment was not functioning properly
- The pasteurized milk has been contaminated by raw milk.

This is important because improperly pasteurized milk still could transmit tuberculosis, brucellosis, and Q fever (Volk and Wheeler, 1980).

2.5.9 Other Milk Quality Tests

a. Organoleptic tests

Bacteria cause various undesirable and detectable organoleptic and physical changes in milk. Generally, when actively growing types of organism capable of causing changes in flavor and physical appearance

reach population levels of 5-20 millions per ml; organoleptic and physical changes are evident or imminent (Ashenafi and Beyene, 1994). The general appearance, cleanliness, colour and smell of the fresh milk should be checked at collection before it is blended with milk from other suppliers since the volume and value at risk increases down the chain (Harding, 1999).

b. Sedimentation test

This is Performed by leaving milk in flask or any container and kept for 15-30 minutes and observing if there is any sedimentation of dirt. The sediment can be examined bacteriologically for the presence of bacteria (Warner, 1975).

c. Clot on boiling test

Acidity decreases the stability of milk. If the concentration of hydrogen ion is more than the normal amount (O'Mahony, 1988), then casein will get precipitated on heating immediately. The clot on boiling test is used to determine whether milk is suitable for processing, as it indicates whether the milk is likely to coagulate during processing (usually pasteurization). It is performed when milk is brought to the processing plant. If the milk fails the test, it is rejected (O'Mahony**,** 1988).

d. Catalase test

This measures the activity of the enzyme catalase. The catalase content of milk primarily depends upon the number of cells in milk. Hence the increased activity of this enzyme indicates mastitis (Cheesbrough, 2000).

e. Specific gravity

To test adulteration, specific gravity is measured and calculated. The specific gravity of milk will be measured using lactometer. The specific gravity of normal unadulterated cow's milk is between 1.026 and 1.032 at 20°C (Ombui *et al*., 1995).

f. Freezing test

The normal freezing point of milk is between -0.50 and -0.61°C. The soluble constituents, lactose and ash determine the freezing point of milk and are responsible for its being lower than that of water. This fact makes it possible to determine whether or not milk has been watered. It had been shown that with addition of 1% of water to milk, the freezing point is raised approximately by 0.0055°C (Hansen, 1994).

2.6ANTIBIOTIC SUSCEPTIBILITY

Antibiotics are essential therapeutic tools for a wide variety of illnesses caused by bacterial infections. The rapid emergence of antibiotic resistant pathogens negates effective treatments and therefore is becoming a major threat to public health (Wassenaar, 2005). It is a significant health, social and economic problem at this time.

Infections caused by antibiotic-resistant bacteria often fail to respond to standard treatments, thereby reducing the possibilities of effective treatment and increasing the risk of morbidity and mortality in serious diseases (Martinez, 2009; Collignon *et al*., 2009). In recent years accumulating problems with bacteria that are resistant to antibiotics occur globally (Keyser *et al*., 2008). Evidence obtained from laboratory and epidemiological studies indicates that the persistence of resistant bacteria is related to the persistence of antibiotic use (Andersson, 2003).

The health safety of foods (Mareček *et al*., 2008; Fikselová *et al*., 2008), including milk, is an integral part of consumers policy and health (Bíreš, 2004). Increase in antibiotics resistance indicates the great capacity of bacteria to overcome the antibiotic pressures. Therefore, at a given time, antibiotic resistance will emerge. In view of this, there are no antibiotics to which resistance has not been recorded (Neely and Holder, 1999; Richet *et al*., 2001; Florea and Nightingale, 2004)

Tetracyclines, β-Lactams,Co-trimoxazole, macrolides, fluoroquinolones, aminoglycosides and chloramphenicols are common antibiotics that are used as therapeutics to treat human illnesses (Barton, 2000).Description of profiles of these antibiotics isdiscussed below;

a. Tetracyclines

Tetracyclines consist of a group of antibiotics obtained as byproducts from the metabolism of various species of *Streptomyces*, although some members may now be thought of as being semisynthetic. The tetracyclines are broad-spectrum antibiotics, that is, they have a wide range of activity againstGram-positive and Gram-negative bacteria. They are bacteriostatic and inhibit protein synthesis by preventing the binding of amino aceyltransferase RNA to the ribosomal site. It also chelates magnesium ion to form tetracycline or magnesium complex preventing protein synthesis. The tetracyclines are no longer used clinically to the same extent as they were in the past because of the increase in bacterial resistance (Hugo and Russel, 2000).

b. β-Lactams

These are antibiotics produced by the genus *Cephalosporium*. Structurally, all are based upon the four-membered nitrogen-containing beta-lactam ring that gives these agents their antibacterial activity. They can be divided into four groups; penicillins, cephalosporins, carbapenems and monobactams - on the basis of the molecular structures surrounding and supporting this active

site. A fifth group in clinical use is the beta -lactamase inhibitors that do not have intrinsic antibacterial activity (Thompson and Smith, 2000). They are bactericidal drugs. They inhibit building of bacterial cell wall by interference with the synthesis of peptidoglycan. The bacterial enzymes that are affected by beta-lactams are called penicilin-binding proteins (PBPs). There are various PBPs differing in their detail function, quantity, and affinity for beta-lactams (Matagne *et al*., 1999). Principally, the effect of beta-lactams is mostly expressed against multiplying bacteria that are building their cell wall intensively. The penicillins are large group of bactericidal compounds with a ß-lactam ring fused with a thiazolidine nucleus. Addition of various side chains (R) to the basic penicillin molecule creates classes of compounds with the same mechanism of action as penicillin but with different chemical and biological properties. They can be classed into four groups: natural penicillins (G and V), antistaphylococcal, antipseudomonal and aminopenicillins. The aminopenicillins such as amoxicillin and ampicillinare effective against streptococci, enterococci and some gram negative organisms but have variable activity against staphylococci and are effective against *Pseudomonas aeruginosa* (Wright, 1999).

c. Co-trimoxazole

Co-trimoxazole is a mixture of five parts of sulphamethoxazole and one part of trimethoprim. This combination results in an in vitrosynergistic antibacterial effect by inhibiting successive steps in folate synthesis. Gram positive bacteria are generally or moderately susceptible. Co-trimoxazole is the agent of choice in treatingpneumonia caused by *Pneumocystis carinii* in patients receiving immunosuppressive therapy and in those with AIDS. It has combined effects of its component. Sulphamethoxazole prevents the formation of dihydrofolic acid while trimethoprim acts by interfering with the action of bacterial dihydrofolate reductase, inhibiting synthesis of tetrahydrofolic acid which is necessary for synthesis of DNA precursors (Bean *et al*., 2005).

d. Macrolides

The macrolide antibiotics are characterized by possessingmolecular structures that contain large(12–16-membered) lactone rings linked throughglycosidic bonds with amino sugars.The most important members of this group are erythromycin, oleandomycin, triacetyloleandomycinand spiramycin. The antimicrobial spectrum of macrolides is slightly wider than that of penicillin, and, therefore, macrolides are a common substitute for patients with a penicillin allergy. Beta-hemolytic streptococci, pneumococci, staphylococci, enterococci, *Neisseria* and *H. influenzae* are usually susceptible to macrolides. Unlike penicillin, macrolides have been shown to be effective against *Legionella pneumophila*, mycoplasma, mycobacteria, some rickettsia, and chlamydia. Macrolides are protein synthesis inhibitors. The mechanism of action of macrolides is inhibition of bacterial protein biosynthesis, and they are thought to do this by preventing peptidyltransferase from adding the peptidyl attached to tRNA to the next amino acid as well as inhibiting ribosomal translocation. Another potential mechanism is premature dissociation of the peptidyl-tRNA from the ribosome.Macrolide antibiotics do so by binding reversibly to the P site on the subunit 50S of the bacterial ribosome. This action is considered to be bacteriostatic. Macrolides tend to accumulate within leukocytes, and are, therefore, transported into the site of infection (Tenson *et al*., 2003).

e. Fluoroquinolones

Fluoroquinolones are broad-spectrum antibiotics that play an important role in treatment of serious bacterial infections, especially hospital-acquired infections and others in which resistance to older antibacterial classes is suspected. There are the second generation quinolones in which

their C-6 is substituted with fluorine resulting to increased potency and spectrum of activity compared with nalidixic acid. Fluoroquinolones such as ofloxacin, ciprofloxacin, norfloxacin show superior activity against Enterobacteriaceae and *Ps. aeruginosa*, and their spectrum also includes staphylococci but not streptococci (Andersson and MacGowan, 2003).It functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV, which is an enzyme necessary to separate replicated DNA, thereby inhibiting bacterial cell division. Fluoroquinolones interfere with DNA replication by inhibiting an enzyme complex called DNA gyrase. This can also affect mammalian cell replication. In particular, some congeners of this drug family display high activity not only against bacterial topoisomerases, but also against eukaryotic topoisomerases and are toxic to cultured mammalian cells and *in vivo* tumor models. Although fluoroquinolone is highly toxic to mammalian cells in culture, its mechanism of cytotoxic action is not known (Owens and Ambrose, 2005). Fluoroquinolones can enter cells easily via porins and, therefore, are often used to treat intracellularpathogens such as *Legionella pneumophila* and *Mycoplasma pneumoniae*. For many Gram-negative bacteria, DNA gyrase is the target, whereas topoisomerase IV is the target for many Gram-positive bacteria (Mittmann*et al*., 2002).

f. Aminoglycosides

Aminoglycoside antibiotics contain amino sugars in their structure. Deoxystreptamine-containing members are neomycin, framycetin, gentamicin, kanamycin, tobramycin, amikacin, netilmicin and sisomicin. Aminoglycosides that are derived from bacteria of the

*Streptomyces*genus are named with the suffix *mycin*, whereas those that are derived from *Micromonospora*are named with the suffix *micin*(Kroppenstedt *et al*., 2005).Aminoglycosides have several potential antibiotic mechanisms, some as protein synthesis inhibitors, although their exact mechanism of action is not fully known. They interfere with the proofreading process, causing increased rate of error in synthesis with premature termination.Also, there is evidence of inhibition of ribosomal translocation where the peptidyl-tRNA moves from the A-site to the P-site. They can also disrupt the integrity of bacterial cell membrane as well as bind to the bacterial 30Sribosomal subunit (Shakil *et al*., 2007).Gentamicin is active against many strains of Gram-positive and Gram-negative bacteria, including some strains of *Ps. Aeruginosa*, *Acinetobacter*, and *Enterobacter*.Some species of *Mycobacteria*, including the causative agent of tuberculosis, are also susceptible to aminoglycosides.

Its activity is greatly increased at pH values of about 8. It is often administered in conjunction with a beta-lactam to delay the development of resistance. Gentamicin is the most important aminoglycoside antibiotic; it is the aminoglycoside of choice in the UK and is widely used for treating serious infections. As with other members of this group, side-effects are dose-related, dosage must be given with care, plasma levels should be monitored and treatment should not normally exceed 7 days (Hugo and Russel, 2000).

g. Chloramphenicol

Chloramphenicol is a bacteriostatic antibiotic that became available in 1949 from the culture of *Streptomyces venezuella*. chloramphenicol has a very broad spectrum of activity; it is active

against Gram-positive bacteria (including most strains of MRSA), Gram-negative bacteria and anaerobes (Falagas *et al*., 2008). It is not active against *Pseudomonas aeruginosa*, Chlamydiae, or *Enterobacter* species. It has some activity against *Burkholderia pseudomallei*, but is no longer routinely used to treat infections caused by this organism (it has been superseded by ceftazidime and meropenem). In the West, chloramphenicol is mostly restricted to topical uses because of the worries about the risk of aplastic anaemia. Chloramphenicol is a bacteriostatic drug that stops bacterial growth by inhibiting protein synthesis. Chloramphenicol prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome. It specifically binds to A2451 and A2452 residues in the 23S rRNA of the 50S ribosomal subunit, preventing peptide bond formation.While chloramphenicol and the macrolide class of antibiotics both interacts with ribosomes, chloramphenicol is not a macrolide. It directly interferes with substrate binding, whereas macrolides sterically block the progression of the growing peptide (Lewis *et al*., 1998).

The original indication of chloramphenicol was in the treatment of typhoid, but the now almost universal presence of multiple drug-resistant *Salmonella typhi* has meant it is seldom used for this indication except when the organism is known to be sensitive. Chloramphenicol may be used as a second-line agent in the treatment of tetracycline-resistant cholera.Because of its excellent blood brain barrier penetration (far superior to any of the cephalosporins), chloramphenicol remains the first choice treatment for staphylococcalbrain abscesses. It is also useful in the treatment of brain abscesses due to mixed organisms or when the causative organism is not known (Bhutta *et al*., 1992).

Chloramphenicol is active against the three main bacterial causes of meningitis: *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. It remains the drug of choice in the treatment of meningitis in patients with severe penicillin or cephalosporin allergy and general practitioners are recommended to carry intravenous chloramphenicol in their bag. In low income countries, the WHO recommends that oily chloramphenicol be used first-line to treat meningitis (Lewis *et al*., 1998).

2.6.1Determinants of Resistance in Bacteria

Antibiotic resistance can be classified as either natural resistance or acquired resistance (Todar, 2002). The natural resistance refers to an organism which has the inherent ability for resisting an antibiotic. An example for this is the inherent resistance of a Gram-negative bacterium like *E. coli* to Penicillin G because there is no reaction site of penicillin G in its structure (Je and Kim, 2005). The acquired resistance refers to a qualitative alteration of the genetic material of the organism as the result of microbes changing in some ways to eliminate the effectiveness of drugs through mutation (Rodriguez *et al.,* 2005).

2.6.1.1 Mutation

Exposure of bacteria to sublethal concentrations of antibiotics results in the selection of resistant strains by the process of natural selection. Under continuous antibiotic pressure, the survivor bacteria, which have initial intrinsic resistance to antibiotics, reproduce, spread, rapidly dominate, or can even displace the antibiotic- susceptible population (Silbergeld *et al*., 2008).

Over time, the survivor bacteria undergo mutations which may further enhance their resistance to antibiotics. Spontaneous mutation may lead to the development of antibiotic resistance in

bacteria and favour survival under antibiotic pressure (Conter *et al*., 2009; Silbergeld *et al*., 2008). For example, resistance to fluoroquinolones (FQ) in some bacterial species has been reported to occur spontaneously due to mutations, particularly point mutations, in drug target genes. A single point mutation which occurs in the quinolone resistance-determining region (QRDR) of DNA gyrase A (GyrA), substantially develops resistance towards fluoroquinolones in Campylobacter, while in other enteric organisms (e.g. *Salmonella* and *E.coli*), stepwise accumulation of point mutations has been reported to acquire high-level fluoroquinolone resistance (Luangtongkum *et al*., 2009; Han *et al*., 2008).

2.6.1.2 Gene Transfer

The resistance genes may be acquired by horizontal gene transfer (HGT) which requires a donor of the resistance genes (Martinez, 2009). In bacteria, horizontal gene transfer has been reported mediated by three mechanisms (Matthew, 2007; Luangtongkum *et al*., 2009) namely:

a.Transformation: This is the incorporation of foreign (exogenous) DNA from the surroundings into the genome of a bacterial cell. Transformation may be a main mechanism for acquiring chromosomally encoded resistance (e.g. fluoroquinolone and macrolide resistance in Campylobacter) (Matthew, 2007; Luangtongkum *et al*., 2009). It is a critically important method of gene transfer (Prescott, 2000) *in vitro* but less important *in vivo* (Schwarz *et al*., 2006).

b. Transduction:This is the transfer of resistant genes via a bacterial virus or phage (Schwarz and Chaslus-Dancla, 2001; Matthew, 2007). It is thought to be a relatively unimportant method of resistance transfer because of the specificity of bacteriophages (Prescott, 2000) and the limited

amount of space for DNA to be packaged into the phage (Schwarz *et al*., 2006). Occasionally, resistance plasmids can be accidentally packed up into phage heads during phage assembly and subsequently be able to infect new cells by injecting plasmid DNA into a recipient cell (Schwarz *et al*., 2006). Neither transformation nor transduction requires a viable donor cell or a link between donor and recipient (Guardabassi and Courvalin, 2006).

c. Conjugation:Conjugation is the transfer of resistance genes from a resistant organism to a sensitive organism through a protein channel (Bennett, 1995; Prescott, 2000; Schwarz and Chaslus-Dancla, 2001). Gene transfer in conjugation allows the spread of mobile genetic elements such as plasmids, transposons, or integron/gene cassettes (Hall and Collins, 1995; Bennett, 1995; Schwarz and Chaslus-Dancla, 2001). These elements can possess multiple antibiotic resistant genes and may be responsible for the rapid dissemination of genes among different bacteria (Kruse and Sorun, 1994; Salyers and Amiable-Cuevas, 1997; Sandvang *et al*., 1997). For example, the antibiotic resistant pattern of *S. typhimurium* DT104 constitutes an integron coding for resistance to sulfonamides, ampicillin and streptomycin (Conter *et al*., 2009). Linked clusters of antibiotic resistance on a single mobile element can also aggregate in such a way that antibiotics of a different class or even nonantimicrobial substances like heavy metals or disinfectants can select for antibiotic resistant bacteria (Recchia and Hall, 1997, Salyers *et al*., 2004). Exchange of resistance genes between pathogens and non-pathogens or between gram-positive and negative bacteria has also been documented (Prescott, 2000; McDermott*et al*., 2002; Salyers *et al*., 2004).

2.6.2 Mobile Genetic Elements

The acquisition of genetic elements such as plasmids, transposons, or integrons/gene cassettes has been reported as a critical part of horizontal transfer of antibiotic resistance. These elements vary considerably from each other in regard to their carriage of resistance, their replication and

transmission. It is estimated that mobile genetic elements accounts for more than 95 percent of antibiotic resistance acquired by gene transfer (Silbergeld *et al*., 2008). They transmit genetic resistance determinants for several different antibiotic resistant mechanisms and may result in rapid dissemination of resistance genes among different bacteria (McDermott *et al*., 2002).

2.6.2.1 Plasmids

These are extra-chromosomal circular DNA which can replicate independently, but synchronously with chromosomal DNA (Schwarz *et al*., 2006). When resistance is transferred as a result of plasmids, a copy of the plasmid is always retained by the parent (Cohen, 1993). It has been reported that most plasmids carry the gene required for conjugation, but some plasmids can be mobilized by using the conjugal apparatus for self-transmissible of plasmids within the cell (Marcelo *et al*., 1998).

Plasmids have been reported to code for resistance to one or up to ten different antimicrobials (multiple antibiotic resistance) (Prescott, 2000). Multi-resistant plasmids have been reported as a result of interplasmidic recombination, integration of transposons, or insertion of gene cassettes (Schwarz *et al*., 2006). All resistance genes on a multi-resistant plasmid are transferred when the plasmid is transferred, whether there is selective pressure for all of the resistance genes on the plasmid or for just one of the resistance genes (Schwarz *et al*., 2006). Plasmids have also been reported to act as vectors for transposons and integrons/gene cassettes (Bennett, 1995).

2.6.2.2 Transposons (jumping genes)

These are short sequences of DNA that has been reported to move from plasmid to plasmid, or from plasmid to chromosome and vice versa (Kidwell, 2005). Transposons do not possess replication systems and must be incorporated into chromosomal DNA or plasmids (Schwarz *et al*., 2006). Unlike plasmids, no copy of the transposon remains within the original cell as the transposon moves between the donor and

recipient (Slotkin and Martienssen, 2007). All transposons has been reported to move and integrate into foreign DNA by nonhomologous recombination, which permits the same transposon to be found in the genome or plasmids of highly unrelated organisms (Kazazian, 2004).

2.6.2.3 Integrons

These have been described as mobile element often found on plasmids and are distinct from transposons (Roy, 1995). They are a site specific recombination system that contains an integrase enzyme, a gene-capture site, and a captured gene or genes (Hall and Collins, 1995). The genes are present as mobile gene cassettes that represent small mobile elements that contain only a single resistance gene and a specific recombination site (Recchia and Hall, 1995, Nandi *et al*., 2004). The recombination site allows mobility when they are recognized by site-specific integrases, which catalyze integration of the cassettes at specific sites within the integron thereby permitting integrons containing multiple resistance gene cassettes (Cambray, 2010). Gene expression of an integron is dependent on various factors including promoter strength, gene copy number, the relative distance of the gene cassette from the promoter, and the presence of additional internal promotors (Martinez-Freijo *et al*., 1998, Martinez-Freijo *et al*., 1999). Expression has been reported to be usually mediated via a common promoter situated upstream (5'-end) of the gene cassettes, rather than through individual promoter copies (Matinez-Freijo *et al*., 1998). Higher levels of gene expression have been reported achieved if a second promoter is included adjacent to the first, or if the gene in question is included as multiple copies (Matinez-Freijo *et al*., 1998).

2.6.3 Drivers of Antibiotic Resistance

There are several reported factors which accelerate bacterial antibiotic resistance.

2.6.3.1 Use and Misuse of Antibiotics

Antibiotics are commonly used to treat infections in humans and animals. However, their use and misuse have been reported to exert selection pressure and accelerate selection of resistant bacterial populations. The use and misuse of antibiotics in animal production and human medicine are summarized below.

a. Antibiotics in Animal Production: Antibiotics have been reported used in animal production systems to treat and control bacterial infections as well as for growth promotion (McDermott *et al*., 2002; Conter *et al*., 2009). The prolonged use of antibiotics, particularly at low levels, promotes the selection of antibiotics resistance among commensal bacteria in the gastrointestinal tract of food animals. For example, Fluoroquinolone-resistant *Campylobacter* have emerged as a result of Fluoroquinolone use in poultry (Asai *et al*., 2007). The increasing resistance to quinolones observed in humans has been reported to be as a result of the use of the same class of antibiotics in animals (Neely and Holder, 1999). When contaminated food is consumed, the resistance gene from commensal bacteria has been reported transferred to other bacteria, including foodborne pathogens, in the intestinal tract of humans. Several studies conducted by the Centers for Disease Control and Prevention (CDC) on antibiotic-resistant *Salmonella* showed that increased resistance in *Salmonella* strains was most likely due to the antibiotic use in food animals, and that most infections caused by resistant strains are acquired from the consumption of contaminated food such as milk and meat products (McDermott *et al*., 2002; Gilchrist *et al*., 2007). This increase was due to sequential acquisition of plasmids and transposons coding for drug resistance to a wide range of antibiotics such as ampicillin, chloramphenicol, gentamicin, kanamycin, sulphonamides, tetracycline and trimethoprim (giving rise to R-type ACGKSSuTTm) (Threlfall, 2002). It has been postulated that the higher the prevalence of bacterial resistance in animal production, the greater the extent of transfer of antibiotics

resistance from animals to humans (Van den Bogaard and Stobberingh, 2000; Nel, 2002). In view of this, even in the presence of specific pressure amongst humans, development of bacterial resistance among human bacterial isolates has been reported due to transfer of resistance via members of say, enterobacteriaceae (Nel, 2002). This could possibly explain why persons exposed to farm animals and abattoir workers have a considerably higher percentage of antibiotic resistant *E. coli* in their intestinal flora (Ishihara *et al*., 2001; Nel, 2002; Van den Bogaard and Stobberingh, 2000).

b. Antibiotics in Human Medicine: Antibiotics have been reported commonly used in human medicine to treat bacterial infections. They are not meant to be used against viral infections like common cold, most sore throats, and flu (FDA, 2010; CDC, 2011). Both overuse, such as over-prescribing of antibiotics for critically ill patients, and underuse, such as taking inadequate dose for inappropriate length of time, have been reported as the main cause of selection of antimicrobial-resistant bacteria populations (WHO, 2011; CDC, 2011). The role played by the spread of resistant bacteria from farm animals to humans has been reported as a major factor in the development of resistance among human bacteria isolates (Bonten *et al*., 2001, White *et al*., 2002, Threlfall, 2002, Ungemach *et al*., 2006). The inappropriate use of antibiotics in the hospitals and close contact among sick patients creates an environment for the dissemination of antibiotic-resistant bacteria (Neely and Holder, 1999; NIAID, 2009). For example, methicillin-resistant *Staphylococccus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) are mainly associated with hospital environments or those who have had prolonged stays in the hospital (Hawkey, 2008; Goodyear, 2002).

2.6.3.2 Environmental Stresses

Several environmental stresses, which are frequently applied in food preservation processes, have been linked to the increase in bacterial resistance towards antibiotics. For example, a study reported an increase in antibiotics resistance in foodborne pathogens, including *S. aureus*, *E. coli*, and *S. enterica* serovar Typhimurium, under sublethal low pH or high sodium chloride stress. Another study showed that high osmolarity and starvation regulates the expression of bacterial lipocalin, a protein which helps bacterial adaptation to environmental stress and is responsible for the dissemination of antibiotic resistance genes. Environmental stress can enhance plasmid transfer and plasmid numbers, thereby increasing resistance (McMahon *et al*., 2007).

2.6.3.3 Socio-economic factors

Socio-economic factors have been reported as drivers of bacterial resistance among human bacterial isolates both in developed and developing countries (Byarugaba, 2004). In the latter, antibiotics are available over the counter and hence easily accessible, leading to overuse (Okeke and Adebayo, 2003; Nys *et al*., 2004). This has been reported believed to account for resistance rates of 90% among human bacterial isolates to tetracycline in West Africa where misuse of this group of antibiotics has been practiced for many years (Okeke and Adebayo, 2003). In developing countries, under use has also been identified as an important cause of development of resistance (Byarugaba, 2004, Neely and Holder, 1999). This is because in poorer countries, patients are either unable to afford the full course of the medicines to be cured of their illness, can only purchase counterfeit drugs on black market, or receive sub-optimal doses. In view of this, bacterial resistance would therefore most likely be a problem in Africa where antibacterial use are unregulated and antibiotics are sold often of substandard quality (Richet *et al*., 2001;

Okeke and Adebayo, 2003; Nys *et al*., 2004). The use of substandard antibiotics has been reported to select resistant pathogens during treatment even if the diagnosis is correct (Okeke and Adebayo, 2003; WHO, 2000).

In developed countries, overuse has been identified as the main concern as far as development of bacterial resistance is concerned. This includes subtler ways like prescribing broad spectrum antibiotics when bacteriological evidence indicates that a narrower spectrum drug would be sufficient, and prescribing antibiotics due to patient pressure, when the odds are that the infection is viral, rather than bacterial (Fidler, 1998; WHO, 2002; Okeke, 2005).

2.6.3.4 Role of Antibiotic Residues in Foods of Animal Origin

Humans have been reported to acquire bacterial resistance enteric organisms by ingesting antibiotics treated animal products (Nel, 2002). Antibiotics resistance is a complex problem involving myriad interactions between humans, animals, drug and the environment (Byarugaba, 2004; Williams, 2005). However, out of this complexity a simple truth emerges: antibiotics breed bacterial resistance, no matter the access routes.

2.7 PRIMARY MECHANISMS OF ANTIBIOTIC RESISTANCE DEVELOPMENT OF COMMONLY PRESCRIBED ANTIBIOTICS

2.7.1 Beta-lactams

Resistance to beta-lactam antibiotics has been reported to be mainly due to inactivation by beta lactamases (Livermore, 1995) and decreased ability to bind to penicillin-binding proteins (Georgeopapadakou, 1993). However, beta-lactam resistance may also be a result of decreased uptake of the drug due to permeability barriers or increased efflux via multidrug transporters (Paulsen *et al*.,

1996; Quintiliani *et al*., 1999). The inactivation of beta-lactams has been ascribed to cleavage of the amino bond in the beta-lactam ring by a beta lactamase enzyme (Bush *et al*., 1995; Livermore, 1995; Bush, 2001; Wiegand, 2003). Genes encoding beta-lactamases has been located on either plasmids or the bacterial chromosome (Aarts *et al*., 2006). Examples of specific gene variants for the beta-lactamase family in gram negative bacteria include ampC, tem, shv, oxa and ctx-M (Aarts *et al*., 2006). Extended spectrum beta-lactamases that play an important role in human medicine have also been described (Bradford, 2001), and the AMR genes for methicillin resistant *Staphylococcus aureus* (Aarts *et al*., 2006).

2.7.2 Tetracyclines

Bacterial resistance to tetracycline has been reported to be as a result of the uptake of new genes (Chopra and Roberts, 2001). There are 23 efflux genes reported to code for energy dependent efflux of tetracyclines, 11 ribosomal protection genes which code for protein that protects bacterial ribosomes, 3 genes that code for enzymes that modify and inactivate tetracycline, and 1 gene that have an unknown mechanism (Schwarz *et al*., 2006).

The efflux resistance genes *tet*A, *tet*B, *tet*C, *tet*D and *tet*H are most wide spread reported for gram negative bacteria and are claimed located on transposons (Allmeier *et al*., 1992; Chalmers *et al*., 2000) and plasmids (Schwarz *et al*., 2006). The *tet*B gene confers resistance to both tetracycline and minocylcine, but not to the new glycyclines, while the other efflux proteins confer resistance only to tetracycline (Chalmers *et al*., 2000, Chopra and Roberts, 2001). Resistance to minocycline and glycyclines are relevant as they are newer drugs that play a role in human medicine. The methodologies utilized to identify these different *tet* resistance genes have been described (Fech and Scjwarz, 2000; Kehrenberg *et al*., 2001; Aminov *et al*., 2001; Ng *et al*., 2001; Guerra *et al*., 2004).

Ribosomal protection genes are a second important way for bacterial resistance to tetracycline development. They have been reported to be of gram positive origin but can also be found in gram negative genera (Schwarz *et al*., 2006). An example of a ribosomal protection gene is the *tet*M gene which has a wide range of hosts and is located on a conjugative transposon (Flannagan *et al*., 1994; Chopra and Roberts, 2001; Salyers *et al*., 1995). Other less well described mechanisms of tetracycline resistance include enzymatic inactivation, 16S rRNA mutation, other mutations, and multidrug transporters (Schwarz *et al*, 2006).

2.7.3 Quinolones and Fluoroquinolones

Quinolones and fluoroquinolones are potent inhibitors of bacterial DNA replication (Schwarz *et al*., 2006). The two major mechanisms of bacterial resistance development to fluorquinolone antibiotics have been reported to be by point mutations and decreased intracellular accumulation (Schwarz *et al*., 2006). Several recent reviews deal with the molecular basis and epidemiology of quinolone resistance in *E. coli* and *Salmonella species* of animal origin (Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1999; Bager and Helmuth, 2001; Cloeckaert and Chaslus-Dancla, 2001; Webber and Piddock, 2001; Ruiz, 2003).

Briefly, point mutations in the target genes *gyr*A and *gyr*B coding for DNA gyrase and or for *par*C and *par*E coding for DNA topoismerase IV are frequent in quinolone and fluoroquinolone resistance (Schwarz *et al*., 2006). Detection of these point mutations in the region of the *gyr*A, *gyr*B, or *par*C and *par*E genes can be accomplished through PCR (Aarts *et al*., 2006) while microarrays have been used to assess multidrug efflux systems. Resistance genes associated with multidrug efflux pumps vary depending on the organism involved (Schwarz *et al*., 2006) and they may lead to high levels of resistance to quinolones and other antibiotics where multidrug efflux pumps and decreased membrane permeability are involved (Lee *et al*., 2000). Quinolone and fluorquinolone resistance has also been reported to result from

interaction between different resistance mechanisms, decreased drug uptake and DNA gyrase protection (Schwarz *et al*., 2006)

2.7.4 Aminoglycosides and Aminocyclitols

The main mechanism for aminoglycoside resistance has been reported to be by enzymatic inactivation (Shaw *et al*., 1993; Mingeot-Leclercq *et al*., 1999), but reduced uptake and chromosomal mutations conferring high levels of resistance to streptomycin have also been described (Quintiliani *et al*., 1999). Aminoglycoside resistance has been reported mediated by more than 50 aminoglycoside modifying enzymes that are classified as either aminoglycosiden acetyltransferases (*aac*), aminoglycoside adenyltransferases (*aad* or *ant*), and aminoglycoside phosphotransferases (*aph*) (Shaw *et al*., 1993, Mingeot-Leclercq *et al*., 1999, Aarts *et al*., 2006). Most *aac,* ant and *aph* genes are located on mobile genetic elements such as plasmids, transposons, or gene cassettes (Shaw *et al*., 1993; Recchia and Hall, 1995; Davies and Wright, 1997; Mingeot-Leclercq *et al*., 1999; Sandvang and Aarestrup, 1997). The modifications of aminoglycosides and aminocyclitols by inactivating enzymes have been described in detail in various reviews (Shaw *et al*., 1993, Davies and Wright, 1997).

2.7.5 Chloramphenicol and Florfenicol

Both enzymatic and non-enzymatic chloramphenicol and florfenicol resistance genes have been described (Aarts *et al*., 2006), but enzymatic inactivation has been reported to be the predominant method (Murray and Shaw, 1997; Schwarz *et al*., 2004) of resistance development. Enzymatic resistance genes primarily encoding acetyltranfereases are reported to be the *cat* genes (Aarts *et al*., 2006). Non-enzymatic gene coding for bacterial resistance against chloramphenicol and florfenicol include the *cml* genes on transposon TN1696 and the *flo*R gene (Aarts *et al*., 2006). Efflux systems conferring resistance to chloramphenicol alone or in combination with florfenicol (Schwarz *et al*., 2004), permeability barriers,

and multidrug transporters (Paulsen *et al*., 1996, Schwarz *et al*., 2004) as well as other minor mechanisms of resistance have also been identified for this class of antibiotics (Schwarz *et al*., 2006). Details on different genes and mechanisms for chloramphenicol resistance are available (Schwarz *et al*., 2004)

2.7.6 Sulphonamides and Trimethoprim

Sulphonamides and trimethoprim are competitive inhibitors of different enzymatic steps in folate metabolism (Schwarz *et al*., 2006). Sulphonamide resistance has been reported due to chromosomal mutations in the dihydropteroate synthase (*fol*P) gene or by acquisition of resistant dihydropteroate synthase genes (*sul* genes) (Aarts *et al*., 2006; Schwarz *et al*., 2006). Three *sul* genes have been described in gram negative bacteria (Swedberg and Skold, 1980; Radstrom and Swedberg, 1988; Aarts *et al*., 2006). The *sul*I gene is associated with class 1 integrons and, therefore, often linked to other bacterial resistance genes. It has been reported to be common in gram negative bacterial species as part of transposons or as conjugative plasmids (Sundstrom *et al*., 1988). The *sul*II gene often occurs with streptomycin resistance genes *str*A and *str*B on conjugative or nonconjugative plasmids (Radstrom and Swedberg, 1988; Kehrenberg and Schwarz, 2004), while the *sul*III gene has been found on conjugative plasmids (Perreten and Boerlin, 2003)

Trimethoprim resistance is primarily mediated by acquisition of *dfr* gene encoding resistant dihydrofolate reductase (Aarts *et al*., 2006; Schwarz *et al*., 2006). Transferable trimethoprim resistance has been identified in a variety of gram negative bacteria and several of these genes are part of plasmids, transposons, or gene cassettes (Recchia and Hall, 1995; Ito *et al*., 2004). Other potential mechanisms of trimethoprim resistance for some bacteria include permeability barriers and efflux pumps (Kohler *et al*., 1996; Huovinen, 2001) and *dhfr* and folate auxotrophy (Quintiliani *et al*., 1999). Mutations in chromosomal genes have also been observed (Huovinen, 2001).

These myriad methods of resistance genes transfer in bacterial pathogens has made microbiological quality of food such as milk a critical issue to guarantee the good health of the milk consumers. The study of milk bioburden in Zaria was designed to highlight the milk consumer microbiological challenges in this locality, that is, Zaria, Nigeria.

CHAPTER THREE

MATERIALS AND METHODS

3.1MATERIALS

3.1.1 Equipments

The following equipments were used in this study:

- Incubator (NAPCO Model 630 Portland, Oregon, USA)
- Hot air oven (Townson/Mercer Ltd. Croydon, England)
- Refrigerator (Haier thermocool)
- Autoclave (Yamato, USA)
- Colony counter (Stuart, UK)
- Microscope (Wild Heerbrugg M11, Switzerland)
- Agarose gel unit (HE 33; Hoefer, San Francisco CA, USA)
- UV Transilluminator (302 nm) (Vilber Lourmat, Germany).

3.1.2 Media

Bacteriological media suchNutrient broth, Nutrient Agar, Eosin Methylene Blue Agar, Salmonella Shigella Agar, Mannitol Salt Agar, MacConkey Agar, Methyl Red Voges Proskauer, Simmon's Citrate Agar, Urea Agar Base, Mueller Hinton Agar and Peptone Water were products of Oxoid Ltd., England, while Cetrimide Agar andTriple Sugar Iron Agar were from Sigma chemical Ltd., England. Others such asLuria-Bertani medium was from Difco Ltd., USA.

GeneJet™ Plasmid Miniprep kit was obtained from Inqaba biochemical Industries Pty Ltd, South Africa.

3.1.3 Reagents

Chemical reagents such as Lugol's iodine, Bromocresol purple, Acridine orange, Ethidium bromide, Glycerol, Ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma chemical Ltd., England. Mannitol, Sucrose, Lactose, Glucose, Crystal Violet, Acetone and Carbon fuschin were from BDH Chemicals Ltd., England, whileAgarose gel was from Schwarz/Mann Biotech.Oxidase reagent was obtained from Hopkin and William, London.

3.2. METHODS

3.2.1 Study Area

Samplings for this study were taken randomly from five locations in Zaria, namely Samaru, Sabon-Gari, Tudun Wada, Wusasa and Zaria city.

3.2.2 Media Preparation

Each bacteriological medium used was prepared from commercially available powder according to the Manufacturer's instructions. Sterilization was by autoclaving at 121°C for 15 minutes.

3.2.3 Sampling

Four brands of liquid pasteurized milk were selected from eight brands of packaged milk commonly sold in Zaria. Brands 1 and 2 were in plastic bottles while brands 3 and 4 were in paper packs and were all stored at room temperature at time of purchase. The four brands of milk samples used in this study were all manufactured in Lagos State, Nigeria. A total of two hundred

(200) packaged pasteurized milk samples were bought from five major markets in the locations with different batch numbers, date of manufacture and expiry dates. The four brands of packaged milk samples were manufactured in August, September and October, 2010 while their expiration dates did not exceed May, 2011. Ten packs of four brands of milk samples were bought making a total of forty samples per market. The samples were transported to the laboratory for bacteriological analysis.

3.2.4 Isolation of Organisms

The milk packs were swabbed with 70% ethanol before opening. Using sterile syringe, 1.0ml of milk sample was aseptically withdrawn from the packages to make ten-fold serial dilutions using sterile normal saline. Using pour plate method, 1.0 ml of appropriate diluted sample was mixed with 19.0 ml of melted nutrient agar (40°C) and poured into sterile plates aseptically. The plates were incubated at 37°C for 24 hours. Total viable counts were carried out on nutrient agar plates using colony counter. The numbers of colony forming units (CFU) per millilitre were counted and recorded after 24 hours. Viable colonies were aseptically picked from nutrient agar plates and purified using prepared sterile nutrient broth. Microscopic examination of the selected colonies was carried out to determine cell morphology and Gram staining reactions of the bacterial isolates.

3.2.5 Selective Plating and Identification of Isolates

Isolation of specific bacteria was done by streaking on selective media. Overnight cultures were grown on nutrient broth and a loopful of inoculum from nutrient broth was streaked on selective agar and incubated at 37°C for 24 hours. Mannitol salt agar (MSA) was used for isolation of *S. aureus*, Cetrimide

agar for isolation of *Pseudomonas species*, Eosin Methylene Blue agar for *E. coli*, Salmonella Shigella Agar for *Salmonella* speciesand MacConkey agar for isolation of other Enterobacteriaceae present in the milk sample. On MSA, colonies that appeared yellowish were presumptively identified as Staphylococci while greenish colonies on cetrimide agar were identified as *Pseudomonas* species. Colonies that produced greenish metallic sheen on EMB were presumptively regarded as *E. coli* while colourless colonies with black spot on SSA were identified as *Salmonella* species. Based on lactose fermentation properties Enterobacteriaceae organisms were isolated on MAC agar.

Presumptively identified organisms were sub-cultured on nutrient agar slant, incubated at 37°C for 24 hours and stored in refrigerator at 4°C pending further studies.

3.2.6 Biochemical Test

Identification of bacterial isolates were confirmed by biochemical tests such as Indole, Methyl Red, Voges-Proskauer and Citrate (IMVIC), triple sugar iron, catalase, oxidase, coagulase, urease and sugar fermentation tests following standard methods (Cowan and Steel., 1993). The purity of the isolates was ascertained by plating on the different selective agar before carrying out biochemical tests. Colour changes were observed and recorded using Cowan and Steel manual, and other methods for bacteria identification (Barrow and Feltham, 1993; De silva *et al*., 2001; Ellis and Goodacre, 2006).

3.2.6.1 Indole test

Pure bacterial culture was grown in sterile peptone broth for 24 hours at 37°C. Following incubation, 5 drops of Kovac's reagent was added to the tubes. A positive indole test is indicated by the formation of a pink to red color in the reagent layer on top of the medium within seconds of adding the reagent.

3.2.6.2 Methyl Red-Voges Proskauer test

This test uses MRVP broth. Bacteria isolates were grown in MRVP broth for 24 hours at 37°C After growth, the broth was separated into two different tubes, one for the Methyl Red (MR) test and one for the Voges-Proskauer (VP) test. The pH indicator Methyl Red was added to one tube and a red color indicated positive test. The VP test uses alpha-naphthol and potassium hydroxide to indicate a positive or negative test.

3.2.6.3 Citrate test

This test uses Simmon's citrate agar to determine the ability of a bacteria to use citrate as its sole carbon source. Bacteria colonies are picked up by a straight wire and inoculated into slope of Simmons citrate agar and incubated overnight at 37 °C. If the organism has the ability to use citrate, the medium changes its color from green to blue.

3.2.6.4 Triple sugar iron test

The TSI agar slants were inoculated with pure culture by streaking over the entire surface of the slant and then stabbing deep into the butt. This was incubated at 37ºC for 24 hours.Glucose fermentation was indicated by the butt of the slants becoming yellow and the slant remaining red (K/A).Glucose, Lactose and Sucrose fermentation was indicated by both slant and butt becoming yellow in TSI agar A/A. No color change indicated that no sugar was fermented. The development or appearance of one or several bubbles in the butt indicated gas formation.Formation of H_2S was determined by the blackening of the whole butt or a streak or ring of blackening at the slant butt.

3.2.6.5 Catalase test

A drop of 3% hydrogen peroxide solution was placed on a glass slide. A bit of growth was then removed from the solid medium with a wire loop and emulsified in the hydrogen peroxide. A positive test was indicated by prompt bubbling and frothing.

3.2.6.6Oxidase test

Two drops of 1% freshly prepared oxidase reagent (phenylenediamine) was placed on a filter paper in a clean Petri dish. The test organism was smeared on it with a glass rod. A positive result showed deep purple colour appearing within 5-30secs. The absence of deep purple colour indicates a negative result.

3.2.6.7 Coagulase test

A drop of physiological saline was placed on a clean glass slide and a colony picked from the solid medium was emulsified in the saline. A loopful of citrated human plasma was added to the bacterial suspension and mixed using the wire loop. The slide was then held up and tilted back and forth for one minute. A positive test is indicated by clumping of cells in the mixed suspension.

3.2.6.8 Urease test

Urea agar base was prepared according to the manufacturer's instructions and slants were made in test tubes. A heavy inoculum from an 18 hour pure culture was streaked on the entire slant surface. The slant was incubated at 37°C for 24 hours. The development of a deep red colour with ammonia fumes indicates a positive reaction.

3.2.6.9 Sugar fermentation test

This uses Phenol Red Broth to test for the fermentation of different sugars. Phenol Red Broth is a general purpose fermentation media that includes the pH indicator Phenol Red and a series of tubes each with a different sugar. A wire loop was aseptically used to inoculate the test organism into the broth containing the test sugar and inverted Durham tubes. This was incubated at 37°C for 18 hours. A bright yellow colour indicated the production of enough acid products from fermentation of the sugar. Production of gas was determined with a Durham tube, a small inverted vial filled with the broth. Gas production during fermentation of the sugar, was seen trapped at the top of the Durham tube and appeared as a bubble.

3.2.7 Antibiotics Susceptibility Test

This was performed using a panel of 12 antibiotics using the disk diffusion method according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2002).

An overnight culture of each isolate was prepared on nutrient broth and incubated at 37°C for 18 hours. Dry sterile plates of prepared Mueller Hinton's agar were inoculated with the standardized inoculums of 18 hours culture test bacteria isolate. Gram negative bacterial isolates were standardized to 10^5CFU/ml while Gram positive bacteria isolates were standardized to 10^6CFU/ml (Jorgensen and Turnidge, 2007). After inoculation, plates were allowed to dry in sterile incubator at 37°C before placing the sensitivity multi-disc, of various antibiotics aseptically in triplicate. Antibiotics impregnated disk from (ABTEK, UK) used include; Amoxycillin (25µg), Amoxicillin/clavulanic acid (30µg), Chloramphenicol (30µg), Co-trimoxazole (25µg), Cloxacillin (5µg), Cefuroxime (30µg), Erythromycin (5µg), Gentamicin (10µg), Nalidixic acid (30µg), Nitrofurantoin (300µg), Ofloxacin (30µg) and Tetracycline (10µg). Plates were allowed to stay for one hour before incubating at 37°C for 18 hours. The zones of inhibition were measured to the nearest millimeter using a transparent ruler.

3.2.8 Determination of Minimum Inhibitory Concentrations (M.I.C.)

The M.I.C. of amoxicillin, chloramphenicol, ofloxacin and tetracycline were determined using agar plate dilution methods as described by Lennette *et al*. (1990) with modifications.

Graded concentrations (starting from 1000µg/ml and 100µg/ml) of the various antibiotics in 10.0 ml volume were prepared in triplicates using sterile distilled water. This was aseptically mixed with 10.0 ml volume of double strength Mueller Hinton's agar and allowed to set. Ten (10) ml volume of sterile distilled water mixed with 10 ml double strength Mueller Hinton's agar was set up as positive control. Eighteen hours broth culture of each test bacteria isolate was standardized to inoculums density of 10^5CFU/ml (Wood and Washington, 1995). The dried Mueller Hinton's agar surfaces were aseptically inoculated with twenty microliter of standardized test organisms in triplicates at equidistant spacing. The plates were allowed to stay for one hour and then incubated at 37°C for eighteen hours. The plates were examined for the presence or absence of growth after incubation period. The lowest antibiotic concentration at which there was no visible growth was taken as the minimum inhibitory concentration (M.I.C.).

3.2.9 Beta- Lactamase Production Test

This was performed using rapid acidometric filter paper method as described by Cheesbrough (2000). Emulsified cell suspensions were made with sterilized loop in triplicate from an overnight nutrient agar slant culture of resistant isolates. A strip of Whatman number one filter paper were placed in the bottom of a sterile Petri-dish followed by a few drops of buffered crystalline penicillin bromocresol purple solution until the paper was almost saturated. Using a sterile wireloop, the emulsified cell suspension (10-20 colonies) of test organism was spread on the filter paper, covering an approximately

5mm in diameter. The lid of the petri dish was replaced and incubated at 37°C for one hour. Known positive and negative beta-lactamase producing strains of *S. aureus* and *Proteus species* were used and examined alongside the test organisms. A yellow colour on the filter paper strip indicated the production of penicilloic acid from the breakdown of penicillin by a beta-lactamase producing organism.

3.2.10 Conjugation Studies

The transfer of resistant traits by resistant isolates to ofloxacin sensitive *E. coli* and *Proteus vulgaris* were investigated using the methods described by Onaolapo *et al*. (1997) with some modifications. The minimum inhibitory concentrations (M.I.C.) of the test antibiotics against the sensitive isolates were determined. The resistant isolates were grown in 5ml sterile nutrient broth and incubated at 37°C for eighteen hours. The ofloxacin sensitive *E. coli* and *Proteus vulgaris* were subcultured into 5ml sterile nutrient broth and incubated at 37°C for eighteen hours. The overnight cultures of the potential donor (R^+) i.e. resistant isolates and recipient (R^-) i.e. sensitive isolates were grown in a ratio of 1:10 respectively in 5 ml volume of sterile nutrient broth, incubated in a thermostatic incubator at 37°C for eighteen hours. Loopfuls of transconjugant from the test organism admixture bottles were subcultured in triplicates on MacConkey agar plates incorporated with the antibiotics M.I.C. (0.1875µG/ml of ofloxacin) against sensitive isolates and incubated at 37°C for eighteen hours. The plates were examined for the presence or absence of cultural characteristics and lactose fermenting properties of sensitive isolates (*E. coli* and *Proteus vulgaris*). The colonies of transconjugants were aseptically picked and transferred to nutrient agar slant, after which the M.I.C. were determined as described by (Lennette *et al*., 1990).

3.2.11 Curing of Transconjugants

The curing of transconjugants was carried out by treatment with acridine orange dye as described by Onaolapo and Klemperer (1986).

Each of the transconjugants was grown overnight on sterile nutrient broth and incubated at 37°C for eighteen hours in a static incubator. The overnight cultures were standardized to 10^5CFU/ml. A stock solution of acridine orange in sterile distilled water (10,000µg/ml) was prepared and 1.0 ml of the solution was dispensed into test tubes containing 2ml sterile nutrient broth. The content of each tubes were vortexed and mixed properly and allowed to settle. Twenty microliter (20µl) of standardized transconjugants was inoculated into the mixed solution of acridine orange and sterile nutrient broth. This was incubated at 37°C for eighteen hours. The growth from the overnight culture of transconjugants was subcultured on MacConkey agar. Antibiotic susceptibility test using M.I.C. method was carried out on colonies obtained from the MacConkey plates. This was to determine whether the resistant pattern has changed.

3.2.12 Isolation of Plasmid

The transconjugant strains and resistant isolates were subjected to plasmid DNA isolation following the protocol of Bimboim and Doly (1979) and Vogelstein and Gillespie (1979). This test was carried out in Centre for Biotechnology Research and Training (CBRT), Ahmadu Bello University, Zaria.

Each isolate was inoculated into 10ml Luria-Bertani (LB) medium incorporated with appropriate selection antibiotic and incubated for 16hours at 37°C while shaking at 200-250rpm. The bacterial culture was harvested by centrifugation at 8000rpm in a microcentrifuge for two minutes at room temperature. The supernatant was decanted and all remaining medium removed. The pelleted cells

were resuspended in 250µl of resuspension solution and transferred to microcentrifuge tube. Exactly 250 µl of lysis solution was added and mixed thoroughly by inverting the tube 4-6 times until solution was viscous and slightly clear. This was followed by adding 350 µl of neutralization solution and mixed by inverting the tube. Centrifugation was carried out at 10000rpm for five minutes to pellet cell debris and chromosomal DNA. The supernatant was transferred to GeneJET spin column by decanting. Centrifugation was carried out for one minute and the flow-through was discarded. Wash solution of 500 µl was added to the column and centrifuged for 30 to 60 seconds, flow-through was discarded and column placed back into collection tube. The wash procedure was repeated to avoid residual ethanol in plasmid preps. The GeneJET spin column was transferred into fresh 1.5 ml microcentrifuge tube and 50µl of elution buffer was added to the center of the column to elute plasmid DNA. This was incubated for 2 minutes at room temperature and centrifuged for 2 minutes. The purified plasmid DNA was stored at -20°C for further studies.

3.2.13 Agarose Gel Electrophoresis

One percent (1.0 %) agarose gel was used to resolve DNA fragment. This was prepared by combining 1g agarose in ten times concentration of tris acetate ethylene diamine tetraacetate (10ml 10XTAEDTA) buffer and 90ml sterile distilled water in 250ml beaker flask and heating in a microwave for 2 minutes until the agarose is dissolved (Moore *et al*., 2002).

Exactly 0.5 µl of Ethidium bromidewas added to the dissolved agarose solution with swirling to mix. The gel was then poured onto a mini horizontal gel electrophoresis tank and casting combs were inserted. This was allowed to gel for 30 minutes. The casting combs were carefully removed after the gel had solidified completely. One times concentration (1X) TAE buffer was added to the reservoir until it covered the agarose gel.

Precisely 0.5 µl of gel tracking dye (bromophenol blue) was added to 20 µl of each sample with gentle mixing. The sample was loaded onto the wells of the gel at a concentration of 20 µl, the mini horizontal electrophoresis gel setup was covered and electrodes connected. Electrophoresis was carried out at 100-200mA for one hour. At the completion of electrophoresis, the gel was removed from the buffer and viewed under UV-transilluminator. The band pattern of DNA fragments was photographed with a Polaroid camera and documented using electrophoresis gel documentation system. This test was carried out in Centre for Biotechnology Research and Training (CBRT), Ahmadu Bello University, Zaria.

CHAPTER FOUR

4.0 RESULTS

4.1 ISOLATION AND IDENTIFICATION

The bacterial load from packagedmilk samples obtained in various locations such as Samaru, Sabo Gari, Tudun Wada, Wusasa and Zaria City were 1.10 – 19.20 x 10^6 cfu/ml; 1.20 – 17.20 x 10^6 cfu/ml; 1.80 – 14.80 x 10^6 cfu/ml; 1.10 – 16.80 x 10^6 cfu/ml; 1.40 – 19.40 x 10^6 cfu/ml while the mean counts were 5.98 ± 4.0 x 10^6cfu/ml, 3.98 ± 2.7x 10^6cfu/ml, 4.45 ± 3.1 x 10^6 cfu/ml, 4.08± 4.7 x 10^6 cfu/ml and 3.32 ± 4.5x 10^6 cfu/ml respectively.

The mean count from the four brands of packaged milk showed that brand 4 had the highest counts while mean count based on location showed that Samaru had the highest count (Table 4.1, 4.2).

Based on cultural, morphological and biochemical characteristics of the organisms isolated, a total of ten (10) bacterial species comprising one hundred and fifty-three (153) isolates were identified in the 200 milk samples studied.

Escherichia coli(13.1%), *Proteus species* (2.6%), *Salmonella specie* (0.65%), *Providencia species* (3.26%), *Enterobacter species* (36.6%), *Citobacter spp* (0.65%), *Klebsiella species* (1.31%) and *Yersinia specie* (0.65%) were bacteria from Enterobacteriaceae Family. Other bacteria isolated were *Pseudomonas species* (37.9%) and *S. aureus* (3.28%) (Table 4.3). Gram's reaction revealed that the isolates were made of predominantly Gram negative rods (96.7%) and few Gram positive cocci in clusters (3.3%). Four different brands of milk products bought from 5 locations were analyzed and some were found to contain some bacterial isolates. A total of 42 (27.5%) isolates were obtained from the first brand of milk

sample, made of predominantly *Pseudomonas species* (Table 4.3). 21.6% isolates were isolated from the second brand of milk sample, made of predominantly *Enterobacter species* (Table 4.3). The third brand of milk sample consisted of 12.4% isolates and was predominantly *Pseudomonas spp.* (Table 4.3), while 38.6% isolates were obtained from the fourth brand of milk sample made of predominantly *Pseudomonas species* (Table 4.3). *Pseudomonas species* (37.9%) was the most frequently isolated organism followed by *Enterobacter species* (36.6%), *and Escherichia coli* (13%). The least isolated organisms includes *Salmonella spp* (0.7%), *Citrobacter spp*. (0.7%), *Yersinia spp*. (0.7%) and *Klebsiella specie* (1.3%) (Table 4.3).

Table 4.4 shows milk sample analysis from five locations in Zaria. The analysis of the data showed that 33.3% of the isolates were from Samaru, 12.4% from Sabo-Gari, 19% from Tudun-wada, 19.6% from Wusasa and 15.7% from Zaria city. *Escherichia coli* were isolated from four of the locations except Sabo Gari. *Proteus spp*, *Salmonella spp* and *Yersinia spp* were found only in Samaru. *Citrobacter spp* occurred only in Tudun-wada while the two *Klebsiella spp* isolated were found in Wusasa and Zaria city. Higher rate of isolation of *Pseudomonas spp* were from Samaru followed by Sabo Gari. *Enterobacter spp* were found to be present in all the locations of study with high rates occurring in samples from Tudun Wada and Wusasa.

Table 4.1: Mean of Total Aerobic Bacterial Counts of four Brands of Packaged Milk Samples in Zaria

Milk Brands	Number of Samples	Packaging Material	Mean Count (Cfu/ml)
Brand 1	50	Plastic Packs	$3.57 \pm 1.8 \times 10^6$
Brand 2	50	Plastic Packs	$3.13 \pm 2.3 \times 10^6$
Brand 3	50	Paper Packs	$1.26 \pm 0.5 \times 10^6$
Brand 4	50	Paper Packs	$9.62 \pm 2.1 \times 10^6$
Mean Total Count			$4.40 \pm 3.6 \times 10^6$

Table 4.2: Mean Count (Cfu/ml) of Brands of Packaged Milk based on Location

Brands	Samaru	Sabo-Gari	Tudun Wada	Wuzaza	Zaria City
Brand 1	5.49×10^6	5.56×10^6	2.67×10^6	2.67×10^6	1.48×10^6
Brand 2	4.43×10^6	2.78×10^6	6.40×10^6	1.38×10^6	0.68×10^6
Brand 3	2.28×10^6	0.82×10^6	1.03×10^6	1.18×10^6	1.00×10^6
Brand 4	11.7×10^6	6.77×10^6	7.71×10^6	11.1×10^6	10.1×10^6
Mean Count	$5.98 \pm 4.0 \times 10^6$	$3.98 \pm 2.7 \times 10^6$	$4.45 \pm 3.1 \times 10^6$	$4.08 \pm 4.7 \times 10^6$	$3.32 \pm 4.5 \times 10^6$

Table 4.3: Distribution of Organisms in different Brands of Packaged Milk Samples

Isolates	Milk Samples				Frequency no. (%)
	Brand 1	**Brand 2**	**Brand 3**	**Brand 4**	
E. coli	3	1	2	14	20 (13.1%)
Proteus spp	3	_	1	_	4 (2.6%)
Salmonella spp	_	_	_	1	1 (0.65%)
Providencia spp	2	_	_	3	5 (3.27%)
Enterobacter spp	6	28	5	17	56(36.6%)
Citrobacter spp	1	_	_	_	1 (0.65%)
Klebsiella spp	1	_	_	1	2 (1.31%)
Yersinia spp	_	_	1	_	1 (0.65%)
Pseudomonas spp	23	4	9	22	58 (37.9%)
Staphylococci spp	3	_	1	1	5 (3.27%)
Total	42	33	19	59	153

Table 4.4: Distribution of Organisms in Milk Samples based on Sampling Locations in Zaria

Isolates	Location				
	Samaru	Sabo Gari	Tudun-wada	Wusasa	Zaria-city
E. coli	3	_	3	6	8
Proteus spp	4	_	_	_	_
Salmonella spp	1	_	_	_	_
Providencia spp	2	2	_	1	_
Enterobacter spp	11	1	16	16	12
Citrobacter spp.	_	_	1	_	_
Klebsiella spp.	_	_	_	1	1
Yersinia spp.	1	_	_	_	_
Pseudomonas spp	26	16	8	6	2
S. aureus	3	_	1	_	1
Total	51(33.3%)	19 (12.4%)	29 (19%)	30 (19.6%)	24 (15.7%)

4.2 **ANTIBIOTICS SUSCEPTIBILITY TESTING**

The susceptibility testing of the bacteria isolates (n = 153) showed that 99.3% of the examined bacteria isolates were sensitive to ofloxacin, 83% to gentamicin and 51.6% to nalidixic acid (Table 4.5).

The frequency of resistance among isolates from the four brands of milk sample showed that brands 1, 2 and 4 were 100% resistant to cloxacillin (Table 4.6). Higher proportion of the bacterial isolates from brand 4 displayed more resistances to test antibiotics than those from other brands. However, bacterial isolates from brand 3 recorded lower proportion of resistance. From the data, there was no resistance to ofloxacin.

Observations also showed high frequency of resistance to cloxacillin among isolates from Samaru, Tudun-wada, Wusasa and Zaria-city. Furthermore, there was 100% resistance against erythromycin among bacterial isolates from Sabo-Gari and Zaria-city. However, ofloxacin was observed to be effective against bacterial isolates obtained in the five locations (Table 4.7).

Table 4.8 shows that bacterial isolates from the milk sample were resistant to a wide range of antibiotics. All *E. coli* (100%) isolated were resistant to cloxacillin and erythromycin. High resistance was also recorded against amoxicillin (90%), tetracycline (90%), amoxicillin/clavulanic acid (85%) and cotrimoxazole (70%). *Proteus spp* were 100% resistant to amoxicillin, amoxicillin/clavulanic acid, cloxacillin, erythromycin and nitrofurantoin. *Salmonella spp* was resistant against chloramphenicol, cloxacillin, erythromycin and tetracycline but sensitive to other test antibiotics. *Enterobacter spp* recorded high resistance against the penicillins and erythromycin. However all the isolates were sensitive to ofloxacin.

Table 4.5: Antibiotics Susceptibility Pattern of Bacterial Isolates from Milk Sample

Antibiotics	% Susceptibility (n= 153)
Amoxicillin	15.03
Amox/clav	16.34
Chloramphenicol	7.20
Co-trimoxazole	32.00
Cloxacillin	0.00
Cefuroxime	17.64
Erythromycin	0.00
Gentamicin	83.00
Nalidixic acid	51.63
Nitrofurantoin	25.49
Ofloxacin	99.30
Tetracycline	9.80

Table 4.6: Frequency of Antibiotics Resistance among Isolates based on the Brands of Milk

Antibiotics	No. (%) of Resistant Isolates Brand 1 (n=42)	Brand 2 (n=33)	Brand 3 (n=19)	Brand 4 (n=59)
Amoxicillin	90.5	72.7	57.9	91.5
Amox/clav	92.9	63.6	36.8	86.4
Chloramphenicol	92.9	84.4	73.7	77.9
Co-trimoxazole	83.3	66.7	36.8	57.6
Cloxacillin	100.0	100.0	94.7	100.0
Cefuroxime	80.9	54.5	31.6	61.0
Erythromycin	97.6	100	94.7	98.3
Gentamicin	7.1	9.1	5.3	1.7
Nalidixic acid	76.2	18.1	26.3	35.6
Nitrofurantoin	76.2	66.7	57.9	72.9
Ofloxacin	0.0	0.0	0.0	0.0
Tetracycline	57.1	87.9	78.9	96.6

Table 4.7: Antibiotics Resistance Profile of Bacterial Isolates from Five Locations

Antibiotics	No. (%) Resistant Isolates Samaru (n=51)	Sabo-gari (n=19)	Tudun-wada (n=29)	Wusasa (n=30)	Zaria-city (n=24)
Amoxicillin	88.2	78.9	55.2	90.0	100.0
Amox/clav	66.7	84.2	69.0	90.0	87.5
Chloramphenicol	96.1	68.4	82.8	76.7	75.0
Co-trimoxazole	70.6	26.3	62.1	60.0	87.5
Cloxacillin	100.0	94.7	100.0	100.0	100.0
Cefuroxime	94.1	36.8	62.1	33.3	45.8
Erythromycin	98.0	100.0	96.6	96.7	100.0
Gentamicin	5.9	5.3	10.3	0.0	4.2
Nalidixic acid	60.8	42.0	27.6	20.0	45.8
Nitrofurantoin	84.3	52.6	58.6	53.3	91.7
Ofloxacin	0.0	0.0	0.0	0.0	0.0
Tetracycline	82.4	68.4	69.0	66.7	100.0

Table 4.8: Distribution of Bacterial Isolates Resistance Based on Zone of Inhibition Produced By Test Antibiotics (%)

Antibiotics	*E. coli*	*Proteus spp.*	*Salmonella*	*Providencia*	*Enterobacter*	*Citobacter*	*Klebsiella spp*	*Yersinia spp*	*Pseudomonas*	*S. aureus*
	n =20	n=4	n=1	n=5	n=56	n=1	n=2	n=1	n=58	n=5
Amc	85	100	0	80	68	100	100	0	84.5	60
Amx	90	100	0	100	73	100	50	100	87.9	100
Chl	65	75	100	60	82	100	100	0	94.8	60
Cot	70	75	0	60	55	100	100	100	74.1	0
Cxc	100	100	100	100	98	100	100	100	100	100
Cxm	30	75	0	80	45	0	0	0	93	40
Ery	100	100	100	100	100	100	100	100	100	40
Gen	5	0	0	20	4	0	0	0	1.7	60
Nal	35	75	0	40	14	0	0	0	70.7	80
Nit	60	100	0	80	55	0	0	0	98.3	0
Ofl	0	0	0	0	0	0	0	0	0	0
Tet	90	75	100	80	73	100	100	100	86.2	80

KEY: Amx= Amoxicillin, Amc= Amoxicillin/clavulanic acid, Chl= Chloramphenicol, Cot= Cotrimoxazole, Cxc= Cloxacillin, Ery= Erythromycin, Gen= Gentamicin, Nal= Nalidixic acid, Nit= Nitrofurantoin, Ofl= Ofloxacin, Tet= Tetracycline.

4.3 MINIMUM INHIBITORY CONCENTRATIONS OF SELECTED ANTIBIOTICS

Table 4.9 shows antibiotic resistance profile of twenty-six isolates based on Minimum Inhibitory Concencentrations determined. Peak plasma level of each tested antibiotics was used as breakpoint to determine resistance. Ofloxacin showed highest activity while isolates were highly resistant to amoxicillin (100%) followed by tetracycline (92%).

The result obtained in this study shows that all isolates from each study area were resistant to amoxicillin (100%). Bacterial isolates from milk samples that were bought from Zaria city were more resistant to chloramphenicol (60%) and tetracycline (80%) than those from the four other locations. Resistance to ofloxacin was generally low within Tudun wada and Wusasa and no resistance was recorded against isolates from Samaru, Sabo Gari and Zaria city (Table 4.10).

Table 4.11 shows that all the isolates were resistant to amoxicillin (100%). High resistance against chloramphenicol and tetracycline were seen to occur among *E. coli* and *Enterobacter species* while no resistance was recorded against *Proteus spp* and *Providencia spp*. Ofloxacin was more effective than other antibiotics tested.

Table 4.12 shows that isolates from the milk sample were found to be multi-resistant with MAR index of at least 0.2. 22.2% of the bacterial isolates had MAR index of 0.7, 16.7% had MAR index of 0.9, while Lower percentage of the isolates (2.2%) had MAR index of 1.0.

Table 4.9: Antibiotics Susceptibility Profiles of Selected Isolates based on their M.I.C and Peak Plasma Levels

Antbiotics	No of Isolates	Resistant NO	% Resistance	Peak Plasma Level (µg/ml)
Amoxicillin	26	26	100	5
Chloramphenicol	26	9	35	10 – 25
Ofloxacin	26	2	8	3 – 5
Tetracycline	26	24	92	4 – 5

Peak Plasma level obtained from Martindale: The Complete Drug Reference (Sean, C.S., 2011).

Table 4.10: Resistance Profile of Selected Isolates from Five Locations in Zaria based on M.I.C

Antibiotics	Peak Plasma Level	Samaru (n=10)	Sabo-Gari (n=1)	Tudun-Wada (n=6)	Wusasa (n=4)	Zaria-City (n=5)
Amoxicillin	5	100	100	100	100	100
Chloramphenicol	10 – 25	30	0	33.3	25	60
Ofloxacin	3 – 5	0	0	17	25	0
Tetracycline	4 – 5	20	_	33.3	50	80

Peak Plasma level obtained from Martindale: The Complete Drug Reference (Sean, C.S., 2011).

Table 4.11: Resistance Pattern of isolates based on Minimum Inhibitory Concentrations of Selected Antibiotics

Antibiotics	***E. coli***	***Proteus spp***	***Providencia spp***	***Enterobacter spp***
	n = 6	**n = 3**	**n = 3**	**n = 14**
Amoxicillin	100	100	100	100
Chloramphenicol	75	0	0	43
Ofloxacin	17	0	0	7
Tetracycline	50	0	0	50

Table 4.12: Multiple Antibiotics Resistance Index of Isolates in Milk Sample (%)

MAR Index	No. of Isolates	Percentage
0.2	6	6.7
0.3	10	11.1
0.4	10	11.1
0.6	14	15.6
0.7	20	22.2
0.8	13	14.4
0.9	15	16.7
1.0	2	2.2

4.4 **BETA-LACTAMASE PRODUCTION TEST**

The result of beta-lactamase production test showed that twenty-six resistant isolates produced beta-lactamase enzyme and they were all resistant to beta-lactam antibiotics used in this study.

4.5 **CONJUGATION STUDIES**

The result of the conjugation studies on Table 4.13 showed that out of the twenty six (26) donor isolates, nineteen were observed to transfer resistance trait and they had the culture characteristics of the recipient *E. coli* and *Proteus spp*. The M.I.Cs of ofloxacin on transconjugants after conjugation were also observed to increase.

4.6 **TRANSCONJUGANTS CURING**

Changes were observed in the sensitivity pattern of tested transconjugants after curing. Table 4.14 shows that the Minimum Inhibitory Concentrations of ofloxacin for each transconjugants decreased when compared to those before curing in a range of one to seven-fold reduction.

Table 4.13A: Minimum Inhibitory Concentration (M.I.C) of Ofloxacin before and after Conjugation

S/N	Isolate Code	M.I.C on MDR isolates before Conjugation (µg/ml)	Recipient Characteristics	M.I.C on Recipient after Conjugation (µg/ml)
1	S5e	12.5	_	_
2	S4e	0.782	+	50
3	S8e	3.125	_	_
4	E46e	0.391	_	_
5	E6e	3.125	+	50
6	E10e	3.125	+	6.25
7	S15e	1.563	+	6.25
8	E8s	1.563	_	_
9	E5s	6.25	_	_
10	E4s	3.125	+	50
11	S48e	6.25	_	_
12	B7e	6.25	+	6.25

Table 4.13B: Minimum Inhibitory Concentration (M.I.C) of Ofloxacin before and after Conjugation

S/N	Isolate Code	Donor M.I.C before Conjugation (µg/ml)	Recipient Characteristics	Recipient M.I.C after Conjugation (µg/ml)
13	E22s	6.25	+	50
14	B36s	1.563	+	12.5
15	S43s	6.25	+	6.25
16	E23e	12.5	+	50
17	B33e	12.5	+	6.25
18	B5s	6.25	+	6.25
19	B23e	6.25	+	50
20	B27s	1.563	_	_
21	E22e	6.25	+	6.25
22	B25e	1.563	+	12.5
23	B41s	1.563	+	6.25
24	S36s	1.563	+	12.5
25	S31s	3.125	+	12.5
26	S44e	1.563	+	6.25

KEY

+ Represents isolate showing pink on MacConkey agar plates.

— Represent isolates that did not transfer resistant traits.

M.I.C.of ofloxacin before conjugation: 0.195µg/ml

Table 4.14: Minimum Inhibitory Concentrations (M.I.C.) ofOfloxacin on Transconjugants

S/N	Donor Bacteria Isolate Code	M.I.C (µg/ml) before Curing	M.I.C(µg/ml) after Curing
1	S4e	50	0.391
2	E6e	50	0.781
3	E10e	6.25	0.195
4	S15e	6.25	0.781
5	E4s	50	6.25
6	B7e	6.25	6.25
7	E22s	50	0.781
8	B36s	12.5	3.125
9	S43s	6.25	3.125
10	E23e	50	3.125
11	B33e	6.25	3.125
12	B5s	6.25	0.391
13	B23e	50	0.781
14	E22e	6.25	3.125
15	B25e	12.5	3.125
16	B41s	6.25	1.563
17	S36s	12.5	0.781
18	S31s	12.5	3.125
19	S44e	6.25	0.781

4.7 **AGAROSE GEL ELECTROPHORESIS**

Plate 4.1 shows agarose gel electrophoresis of plasmid DNA from resistant bacteria isolates. Lanes 5, 7, 9, 11, 12, 13,14,15 and 16 were shown to harbour plasmids of various sizes.

Plate 4.2 shows agarose gel electrophoresis of both resistant isolates and some transconjugants. Lanes 9 and 11 are shown to harbour plasmids.

Plate 4.3 shows agarose gel electrophoresis of plasmid DNAs from transconjugant isolates. Lanes 6, 7 and 16 are shown to harbour plasmids.

Figure 4.1 shows the semi-logarithm plot of the standard marker, 100bp plus DNA ladder versus the distance moved by the ladder. This was used to estimate molecular weight of isolated plasmid DNA.

Table 4.15 shows the estimated molecular sizes of plasmid DNA isolated from resistant isolates and transconjugants. The sizes were between 2512bp and 10000bp. Plasmid analysis yielded seven different plasmid profiles comprising one and two plasmid number. Four of the isolates shared plasmid profile of sizes 3981bp and 3162bp, three isolates shared plasmid profile of size 5623bp, and two isolates shared profile sizes of 10000bp.

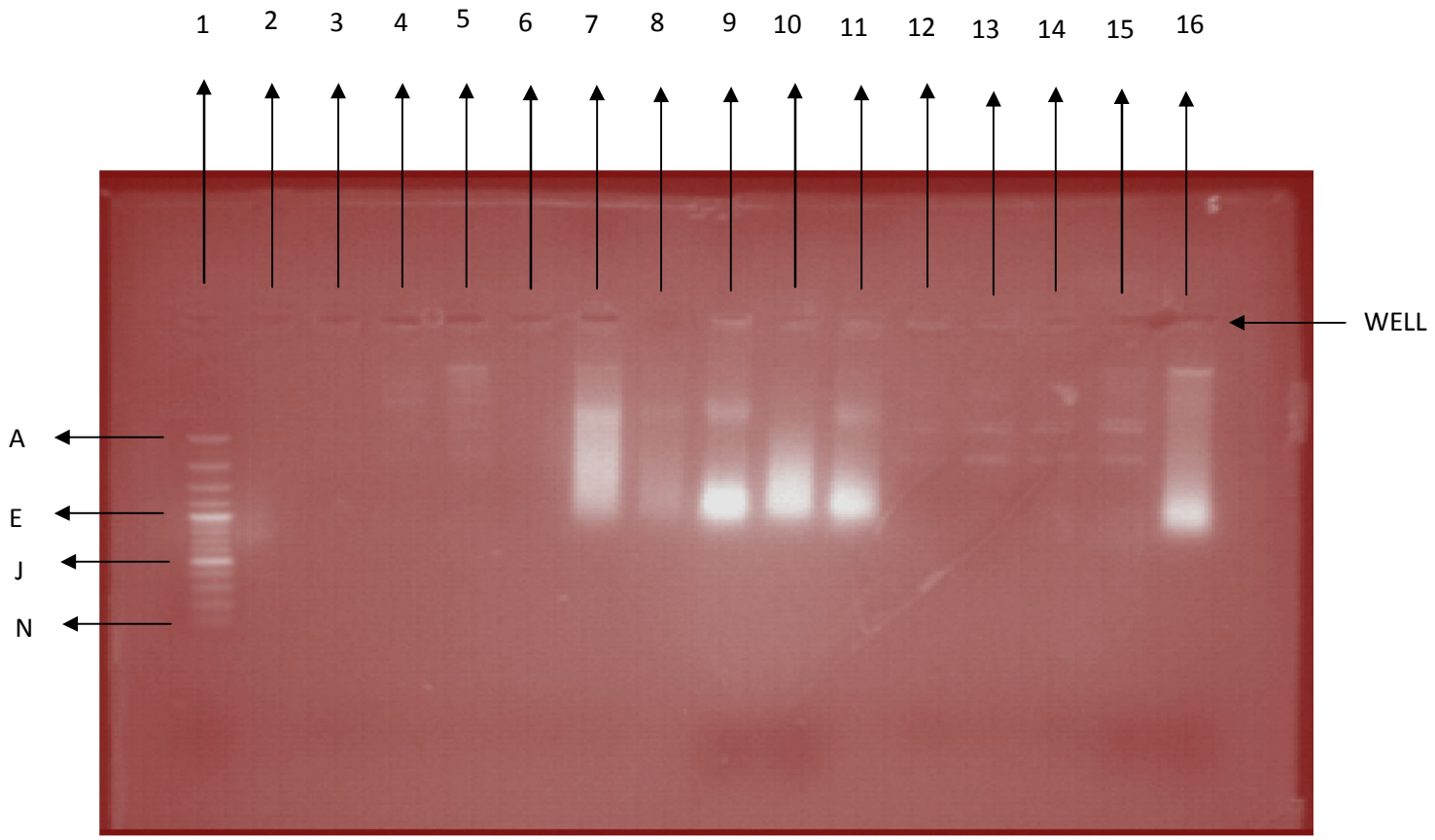


Plate 4.1: 1% Agarose Gel Electrophoresis of Plasmid DNA from Multiple Antibiotic Resistant Isolates.

Lane 1: 100bp Plus Ladder composed of DNA fragments (in base pairs):3000(A), 2000, 1500,

1200,1000(E), 900, 800, 700, 600, 500(J), 400, 300, 200,100(N). Lane 2 to 5: Resistant *E. coli* (Lab nos S5e, S4e, S8e, E46e). Lane 6 to 8: Resistant *Providencia spp* (Lab nos E6e, E10e, S15e). Lane 9 to 11 : Resistant *Proteus spp* (Lab nos E8s, E5s, E4s).Lane 12 to 15: Resistant *Enterobacter spp* (Lab nos. S48e, B7e, E22s, B36s). Lane 16: Resistant *E. coli* (Lab no. S43s).

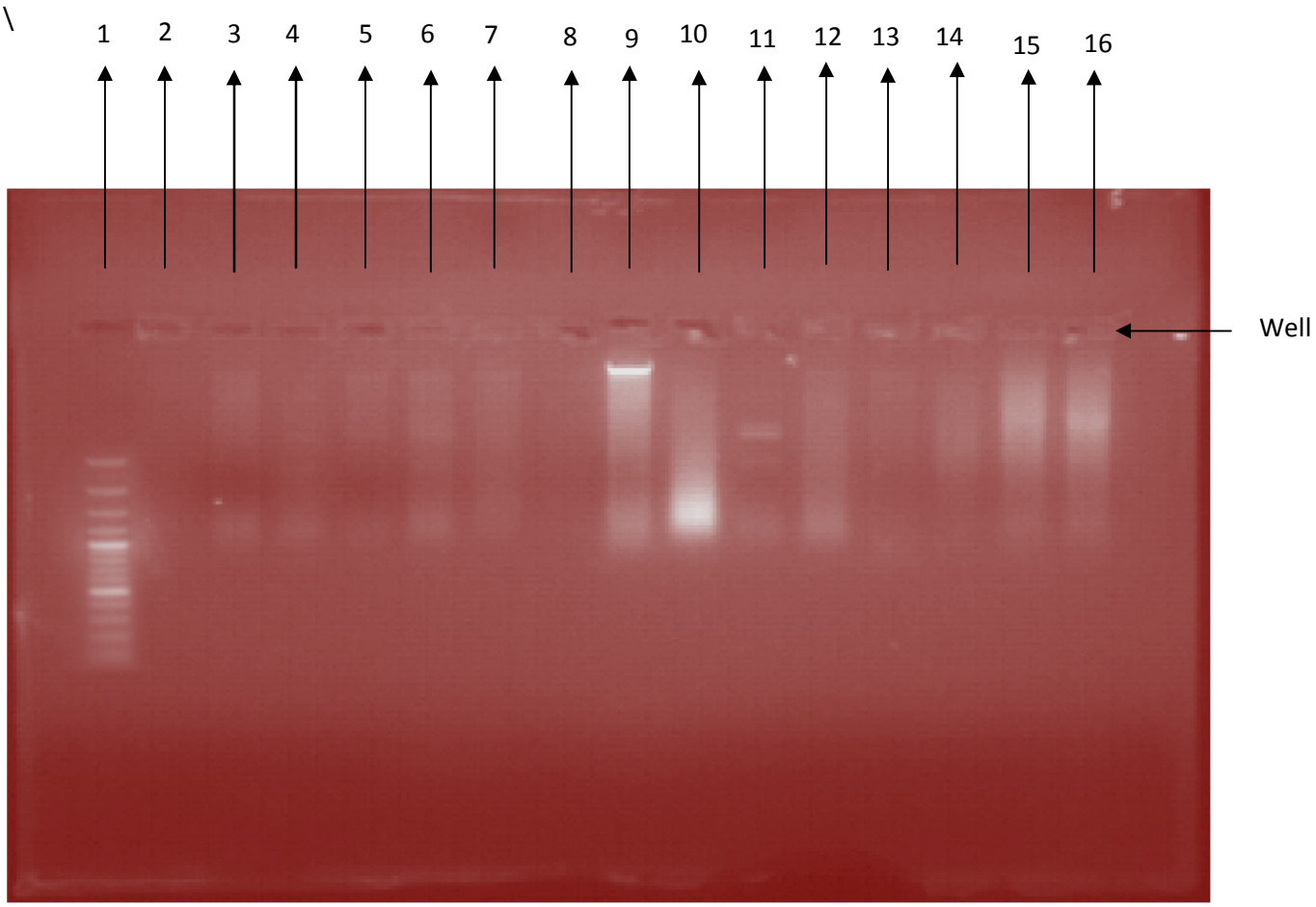


Plate 4.2: 1% Agarose Gel Electrophoresis of Plasmid DNAs from Multiple Antibiotic Resistant Isolates and Transconjugants.

Lane 1: 100bp Plus Ladder. Lane 2: Resistant *E. coli* (lab no E23e). Lane 3 to 12: Resistant *Enterobacter species* (Lab nos B33e, B5s, B23e, B27s, E22e, B25e, B41s, S36s, S31s, S44e). Lane 13 to 16: Transconjugants (isolate lab nos. S4e, E6e, S44e and S15e)

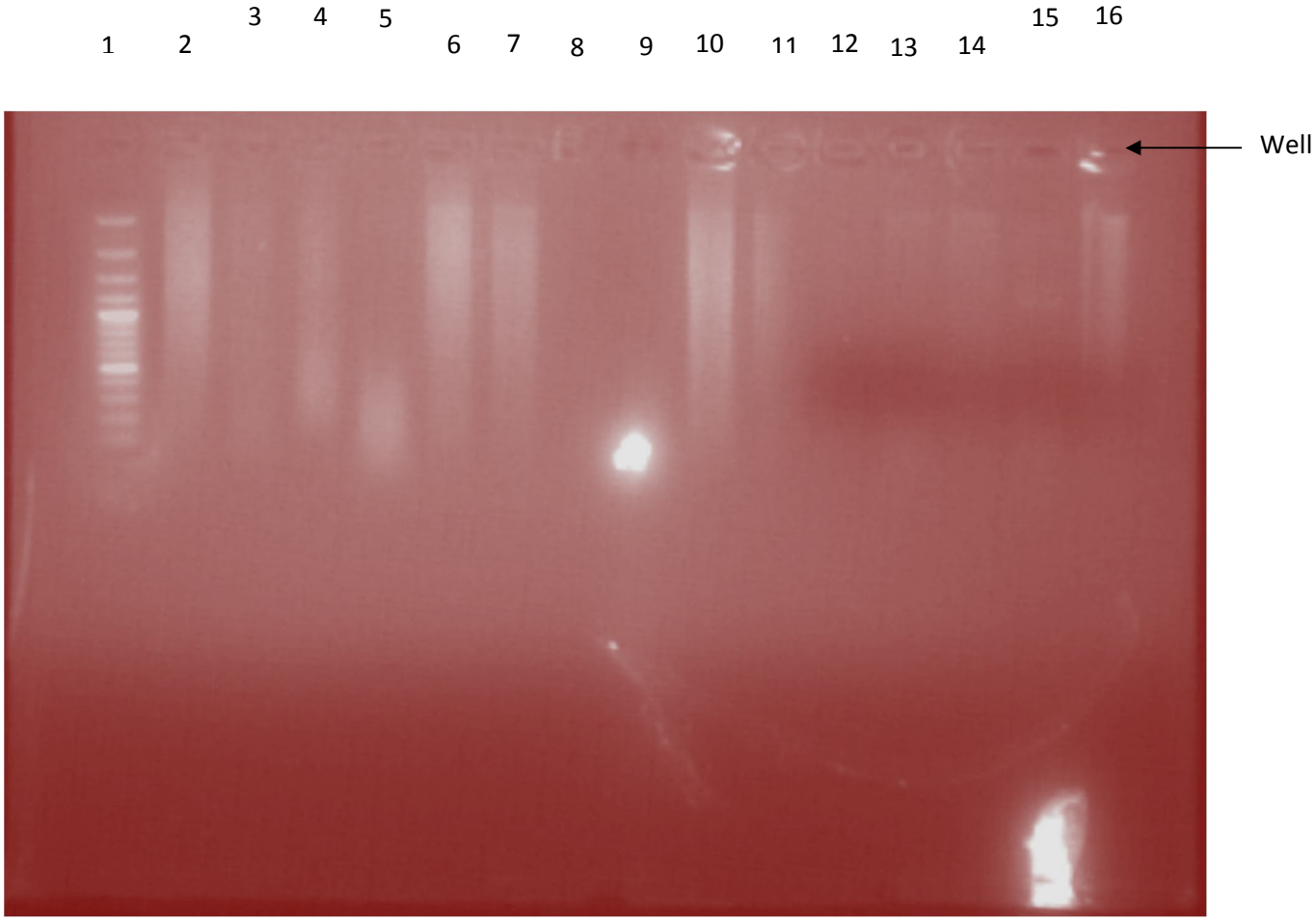


Plate 4.3: 1% Agarose Gel Electrophoresis of Plasmid DNAs from Transconjugant Isolates

Lane 1: 100bp Plus Ladder. Lane 2 to 16: Transconjugants with isolate lab nos. E4s, B7e, E22s, B36s, S43s, E46e, B33e, B5s, B23e, E22e, B25e, B41s, S36s, S31s, E10e.

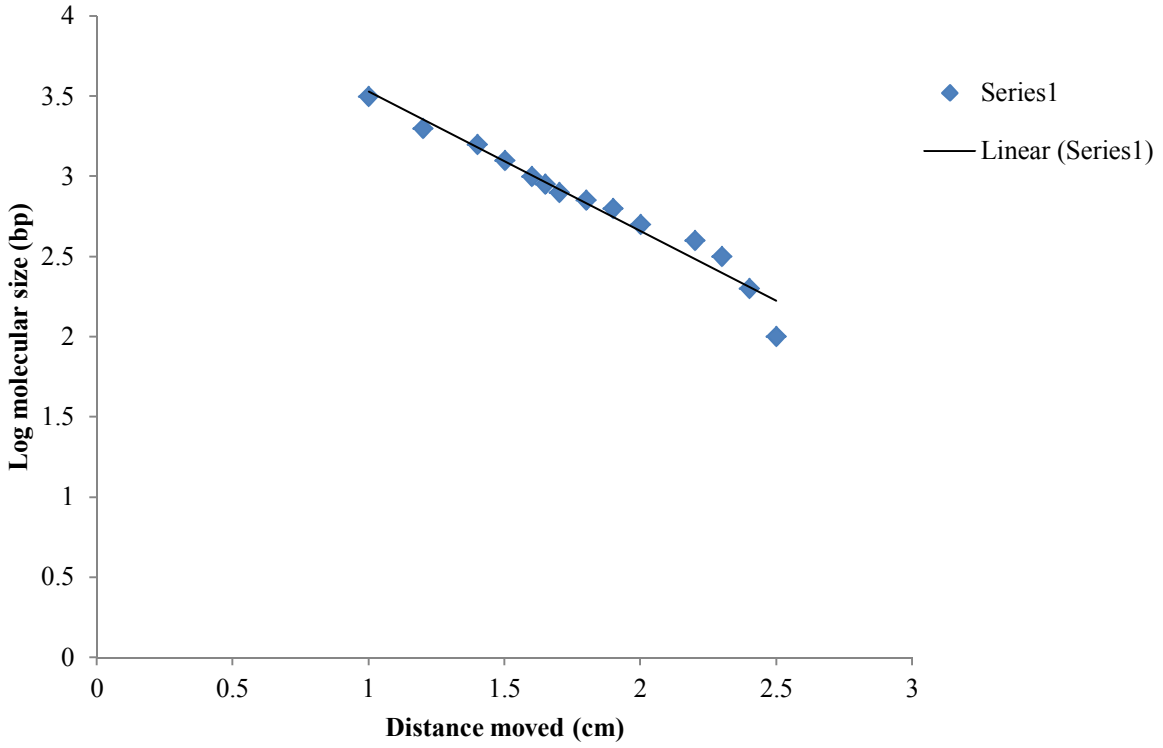


Figure 4.1: Semi-logarithm graph of the molecular weight of the standard DNA ladder vs the distance travelled.

Table 4.15: Plasmid Profile of Isolates Habouring Plasmids

Isolate Code	Isolate	Milk Brand Source	No of Plasmid	Estimated Molecular Sizes in base pairs (bp)
S8e	*E. coli*	4	1	7079
E46e	*E. coli*	1	2	10000, 3548
E10e	*Providencia spp*	1	1	10000, 5623
S15e	*Providencia spp*	4	1	5623
E8s	*Proteus spp*	1	1	5623
E4s	*Proteus spp*	1	1	5623
S48e	*Enterobacter spp*	4	2	3981, 3162
B7e	*Enterobacter spp*	2	2	3981, 3162
E22s	*Enterobacter spp*	1	2	3981, 3162
B36s	*Enterobacter spp*	2	2	3981, 3162
S43s	*E. coli*	4	1	10000
B41s	*Enterobacter spp*	2	1	7079
S31s	*Enterobacter spp*	4	2	3162, 2512
S43s	*E. coli* (Transconjugant)	4	1	10000
E46e	*E. coli* (Transconjugant)	1	1	10000
E10e	*Providencia spp* (Transconjugant)	1	1	10000

CHAPTER FIVE

5.0 DISCUSSION

5.1 Bacterial contamination of milk product sold in Zaria

The results obtained from the bacteriological analysis of milk samples obtained from Samaru, Sabon Gari, Wusasa and Zaria City showed that the products were grossly contaminated with bacteria of public health concern. The bacterial load count from milk products in this study range from 19.4 x 10^6cfu/ml to 1.1 x 10^6cfu/ml. The high total aerobic bacterial counts in the milk products examined could be a consequence of the low level of hygiene maintained during the processing of the milk products. It has been reported that the unclean hands of workers, poor quality of milk, unhygienic conditions of the manufacturing unit and water supplied for washing the utensils could be the source for accelerating bacterial contamination of milk products beside the post manufacturing contamination (El-Mahmood and Doughari,2007). The high numbers of the isolated bacteria observed in this study could be due to the fact that milk being a good nutritive medium enhanced the growth of bacteria contaminant in the mik investigated (International Dairy Federation, 1994a; Adesiyun *et al*., 1997b).

The detection of bacteria from enterobacteriaceae group such as *Escherichia coli,Enterobacter spp*, *Klebsiella spp*, *Proteus spp*, *Citrobacter spp*, *Yersinia spp*, *Salmonella spp* and *Providencia spp* in the studied milk products, probably indicates possible faecal contamination (Talaro and Talaro, 2006). Similar bacteria isolates from milk products have been previously reported from milk products (Yagoub *et al*., 2005; Oranusi *et al.,* 2007)

Being enteric bacteria, their presence indicates poor hygienic practices among handlers of these products. Due to the significance of the faecal-oral route transmission for many bacterial food borne

diseases, basic hygiene measures assume a decisive importance in food safety management (Utermann, 1998). The presence of these bacteria in milk also suggests contamination from various sources, which may include animal, human, environment, utensils and others (Murphy and Boor, 2000).

Isolation of *E. coli* could be due to faecal contaminated water used in milk production, raw materials, storage environment. *Escherichia coli* have been reported linked to diarrheal diseases, urethrocystitis, prostatitis and pyelonephritis (Kurt and Wolfgang, 2000; Leflon-Guiboutae*t al*., 2002).

Other bacteria isolated in this study include *Pseudomonas species* and *Staphylococcus aureus*. *Pseudomonas species* have been implicated in the spoilage of milk and its products at even refrigerator temperatures (Gilmour and Rowe, 1990). *Pseudomonas* has been implicated in localized/generalized infections following surgery or burns, nosocomial infections e.g. Urinary tract infections following catheterization, eye and ear infections which may be serious in hospitalized patients or those with cancer who consume pasteurized milk (Okpalugo *et al*., 2008). Detection of *Pseudomonas spp* can also be due to the low temperature of storage of pasteurized milk, which might have supported the growth of psychrotrophs as reported by Holm *et al.* (2004). Valbuena *et al*. (2004) also reported detection of *Pseudomonas spp* in milk products.

The detection of *Staphylococcus aureus* is also of public health importance because of its ability to cause a wide range of infections especially food-borne intoxication. Isolation of *Staphylococcus aureus* from milk products have been reported in other works (Teale, 2002; Jayarao and Wolfgang, 2003; Sato *et al*., 2004). *Staphylococcus aureus* has been linked to gastroenteritis by producing toxic chemical enterotoxins. As little as 1.0 µg of the toxin in contaminated food produces symptoms of illness. This level of the toxin has been found at 10^5 cells /g of food (Ananthanarayanand Panikaran,2001). *Staphylococcus aureus* has also been reported linked to boils, skin infections, (pneumonia, deep abscesses and meningitis in debilitated persons). *Staphylococcus aureus* has been reported highly

vulnerable to destruction by heat treatment and nearly all sanitizing agents; therefore, the presence of this bacterium in milk is an indication of poor sanitationduring processing, handling and packaging or post pasteurization contamination (Ahmed *et al*., 2009).

5.2. Antibiotics Susceptibility of Bacterial Isolates from Packaged Milk sold in Zaria

Antibiotics susceptibility assessment of bacteria isolates from milk products sold in Zaria, Nigeria in this work showed varying degrees of bacterial resistance as well as multiple antibiotics resistancesin bacterial isolates. Result of antibiotic susceptibility tests on isolates

revealed that most of the isolates were multiresistant to more than three of the antibiotics as also reported in other studies on milk and milk products (Guta *et al*., 2002; Ahmed *et al*., 2001; Okpalugo *et al*., 2008; Nováková *et al*., 2010).The order of antibacterial ineffectiveness of the studied antibiotics generally was Cloxacillin (99%) > erythromycin (98%) > amoxicillin (83%) > chloramphenicol (83%) > tetracycline (81.7%) > amoxicillin/clavulanic acid (77%) > nitrofurantoin (70.6%) > co-trimoxazole (64.1%) > 61.4% to cefuroxime (61.4%) > nalidixic acid (41.8%). Majority of the bacterial isolates obtained from packaged milk products in Zaria, Nigeria were susceptible to ofloxacin (99.3%) and gentamicin (83%) (Table 4.6). The effectiveness of ofloxacin might be attributed to the fact that ofloxacin is a relatively new antibiotic and has not been extensively used to warrant resistance developing against it by pathogens.

The findings from this present study agreed with work reported by Okonko *et al*. (2009a), who reported high bacterial isolates resistance to amoxicillin, tetracycline and cotrimoxazole (60 to 100%). Other workers have reported bacterial isolates obtained from milk to be resistant to cotrimoxazole (Alos *et al*., 2009; Christiaens *et al*., 1998; Oteo *et al*., 2002; Aiyegoro *et al*., 2007). This study highlights a highly diverse antibiotics resistance rates among the bacterial isolates.

Antibiotic resistance of isolated bacteria from milk products may be a reflection of the harmful effects of self medication. Many antibiotics have been reported to be persistent in the environment and have been isolated from ground water (Thurman and Hostetler, 1999) which could probably be used at times in the preparation of milk products. This could enhance the emergence and spread of bacterial resistance among people who may consume these milk products.

In this study, *Klebsiella spp* was 100% susceptible to cefuroxime, gentamicin, nalidixic acid, nitrofurantoin and ofloxacin. However, they were 100% resistant to amoxicillin/clavulanic acid, chloramphenicol, cotrimoxazole, cloxacillin, erythromycin, tetracycline and 50% resistant to amoxicillin. This is a deviation to what has been previously reported (Resih *et al*., 1993; Aiyegoro *et al*., 2007; Okonko *et al*., 2009a). Resih *et al*. (1993) and Aiyegoro *et al*. (2007) reported resistance of 66.7% against amoxicillin and cotrimoxazole and 55.6% to tetracycline by *Klebsiella* spp.

*Escherichia coli*isolates were observed to be resistant to most of the tested antibiotics in this study but 100% susceptible to ofloxacin and 88.9% susceptible to gentamicin. This observation is in contrast with what was reported by other workers who reported sensitivity of *E. coli* isolated from milk to tetracycline and cotrimoxazole (Gupta *et al*., 1999; Aiyegoro *et al*., 2007).

Pseudomonas spp isolated from milk in this investigation showed resistance to majority of the test antibiotics except ofloxacin (100%) and gentamicin (98.3%) which is similar to the findings of Okonko *et al.* (2009a).

5.3 Multiple Antibiotics Resistance Index of Bacterial Isolates from Milk

The multiple antibiotic resistance indices (MARI) give an indirect suggestion of the probable sources of an organism. According to previous workers Krumperman(1983) and Paul *et al*.(1997), MAR index greater than 0.2 indicates that an organism must have originated from an environment where

antibiotics are often used. Multi-antibiotic resistance of three to eight antibiotics was frequent observations in this study among the bacterial isolates. Out of ninety enterobacteriaceae studied82.2% had MAR index of 0.5 and above, while 17.8% had MAR index of less than 0.5. Such multi-antibiotic resistance has important implications for the empiric therapy of infections caused by *Escherichia coli,Enterobacter spp,Klebsiella spp*, *Proteus spp*, *Citrobacter spp*, *Yersinia spp*, *Salmonella spp*, *Ps. aeruginosa*, *and S. aureus* and for the possible co-selection of antibiotic resistance mediated by multi-antibiotic resistance plasmids (Oteo *et al*., 2002; Sherley *et al*., 2004). It has been well documented that gram negative bacilli habour series of antibiotic resistance genes like transposons or integrons and R-plasmids which can be transferred to other bacteria horizontally (Piddock, 2006; Depardieu *et al*., 2007; Leavitt *et al*., 2007; Lockhart *et al*., 2007).

5.4 Beta-lactamase Production of Bacteria Isolates from Milk Sample

Beta-lactamase production investigation revealed that the twenty-six resistant bacterial isolates tested for ß-lactamase enzyme from milk product in this study produced beta-lactamase enzyme capable of hydrolysing beta lactam antibiotics. Akpan, (1992)also reported similar result in Nigeria. This observation confirmed the high beta-lactam antibiotics resistance that was observed against amoxicillin and cloxacillin. The implication of these resistances is that many bacterial diseases that could be treated with inexpensive antibiotics, has recently been made more expensive and less successful by the emergence and spread of resistant organisms (Okeke *et al*., 2007; Okonko *et al*., 2009a, b). However, these multi-antibiotic resistances observed among some of the bacteria isolates from milk products in this study has now become a large and growing problem in infections that account for most of Africa's disease burden, including respiratory and diarrheal diseases (Okeke *et al*., 2007).

5.5 Conjugation Studies of Bacteria Isolates from Milk Product

Resistance genes are often located on extra-chromosomal genetic elements or in segments inserted within the chromosome that originates from other genomes (Carattoli, 2003; Yah *et al*., 2007). The acquisition of a new gene may occur by genetic transformation or through mobilization by conjugative transfer. The latter may occur at high frequency and efficiency, and several resistance genes can be acquired simultaneously (Carattoli, 2003). Plasmid profiles have been reported to be useful in tracing the epidemiology of antibiotic resistance. The result of the conjugation studies suggested possible acquisition of R-plasmids by sensitive *Proteus spp* and *E. coli* from multiple antibiotic resistant isolates. It was obsereved that out of twenty six donor bacteria isolates, nineteen transferred resistance traits to ofloxacin sensitive *Proteus spp* and *E. coli* (Table 4.14). The result of antibiotics susceptibility of the transconjugants using M.I.C method was seen to have changed after conjugation. There was an increase in the M.I.Cs of the bacterial isolates. Changes were observed in the sensitivity pattern of tested transconjugants after curing with acridine dye. Decrease in minimum inhibitory concentration of transconjugants after curing as compared to those before curing revealed that acridine dye was effective curing agent. However, conjugation analysis revealed that apart from plasmids that were transferable by conjugation, other resistance determinants were transferable to sensitive recipient strain of *Proteus* spp and E*. coli* since their M.I.Cs increased. This suggests that these resistance determinants were carried extra-chromosomally on R-plasmids.Similar resistance determinants movement have been attributed to the selection pressure created by uncontrolled use of antibiotics in feed-stuff for animals, in addition to the unregulated use of antibiotics by humans (Aarestrup, 1999; Van den Bogaard and Stobberingh, 2000; Teuber, 2001). Indiscriminate use of antibiotic agents and antibiotic sale behaviour (for example, sale of antibiotics without prescription, sale of under dose and substituting brands) has been reported to enhance the development of antibiotic resistance among pathogenic bacteria (Indalo, 1997). In developed countries, the main reservoirs for antibiotic resistance

in enteric bacteria has been attributed to farm animals such as cattle, sheep, pigs and poultry (CDC, 1996; Pohl *et al*., 1999). Contact with these animals or consumption of food products from them such as milk has been the main route of dissemination of resistance into the human populations.

5.6 Plasmid investigation using Agarose gel electrophoresis

Agarose gel electrophoresis analysis showed the presence of plasmid of various sizes among resistant isolates ranging from 2512 – 10000bp. Some of their corresponding transconjugants contained similar plasmid sizes. Plasmid transfer was observed among *E. coli*, *Proteus spp*, *Enterobacter spp* and *Providencia spp* which were isolated from milk brands 1, 2 and 4. Milk brand 1 displayed high level of bacterial isolates with plasmids; this was closely followed by brand 4. However, brand 2 had only three bacterial isolates that harboured plasmids. The result also showed that out of twenty- six (26) resistant isolates analysed, thirteen (13) were shown to harbour one or two plasmids. Resistant *E.coli* harboured plasmid of sizes 3548, 7079 and 10000bp. Plasmid sizes of *Providencia spp* were 5623 and 10000bp while that of *Proteus spp* were estimated to be 5623bp. This study also revealed that all the *Enterobacter spp* harboured plasmid of sizes of approximately 3162 and 3981bp.

The plasmid profiles observed in this study indicated that the plasmids are distributed at random in these isolates. In most of the cases, bacterial isolates having similar antibiotic sensitivity patterns had different plasmid patterns, implying that plasmid may not have link with the resistance. This supposition can be further supported by the finding that all the plasmidless strains were resistant to one or more antibiotics.

According to some workers Carattoli(2003) and Yah *et al*. (2007), antibiotic resistance in some bacterial isolates which seem not to possess plasmids was associated with chromosome and/or transposons. In determining whether the plasmids resistance markers could be transferred to sensitive isolates, the

results showed that only three of the transconjugants expressed plasmid DNA that migrated approximately on agarose gels. The transferred plasmids DNA varied among the bacterial isolates.Based on resistant phenotypic pattern, bacteria isolates that were resistant to eleven antibiotics harboured plasmid size of 7079bp while bacteria isolates that were resistant to nine or ten antibiotics harboured plasmids that range from 2513 to 10000bp. This shows that there was no relationship between the resistance phenotypic pattern and the plasmid size.

Multiple resistance genes clusters in large plasmids are usually associated with transposons and insertion sequences (Miriagou *et al*., 2006). Plasmid profiles revealed that bacterial isolates with the same resistance profile may differ in their plasmid profiles. This suggests diversity in plasmid contents of bacterial isolates and the contribution of different plasmids in the resistance to a certain antibiotic. The exchange of plasmids between bacterial cells and the integration of resistance genes into specialized genetic elements play a major role in acquisition and dissemination of antibiotic resistance genes among bacteria isolates (Winokur *et al*., 2000; Carattoli, 2003; Helms *et al*., 2004; Osman *et al*., 2006; Yah *et al*., 2007).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION, RECOMMENDATION

6.1 SUMMARY

The major contaminants of packaged milk products analysed were *Pseudomonas spp* closely followed by *Enterobacter spp* and *E. coli* and the overall contamination level of bacterial isolates in this study was 76.5%. Varying degrees of resistance as well as multiple resistances were found in the tested isolates with cloxacillin, erythromycin, amoxicillin, chloramphenicol and tetracycline. However, most isolates were susceptible to ofloxacin and gentamicin. Conjugation analysis revealed that there was transfer of resistance determinants to nineteen bacterial isolates while agarose gel electrophoresis revealed presence of plasmids with various sizes ranging from 2512 to 10000bp.

6.2 CONCLUSION

a. Findings from this study showed that packaged milk samples sold in Zaria, Nigeria were contaminated with bacteria majorly of Enterobacteriaceae group,*Pseudomonas spp* and *S. aureus.*
b. The Bacterial isolates were resistant to test antibiotics in the order of cloxacillin > erythromycin > amoxicillin > chloramphenicol > tetracycline > amoxicillin/clavulanic acid> nitrofurantoin,But sensitive to ofloxacin (99.3%) and gentamicin (83%).
c. Agarose gel electrophoresis analysis revealed presence of plasmids of various sizes ranging from 2512 to 10000bp.
d. Thirteen out of twenty-six resistant isolates harboured one or two plasmids.
e. Plasmidresistance markers could be transferred from the resistant isolates to sensitive isolates as demonstrated by three of the transconjugants S43s, E46e, E10e.

6.3 RECOMMENDATIONS

Based on the results obtained from this study, strict hygienic measures should be applied during production, processing and distribution of milk and its products to avoid contamination by resistant bacteria pathogens.

The key to preventing contamination of milk products is to prevent post-pasteurizationcontamination through well designed quality assurance. It isalso the key responsibility of both consumers and supplierstо adequately store milk at suitable temperatures in order tocontrol the levels of bacteria and to retard the rateof milk spoilage.

Effective measures to ensure safe milk for human consumption such as the phosphatase and methylene blue reduction tests should be routinely performed on each batch of milk processed by dairy plants.

Since public perception of food quality is critical in the marketing of any product, it is very important that the Nigerian milk products industries maintain high processing standards.

Overall, control of all the routes by which multiple antibiotic resistant bacteria and their related genes can arise in the human patient, of which food is but one such route, requires a response from all stakeholders to acknowledge their responsibilities for preventing both the development and spread of such bacteria, each in their own area of activity including medicine, veterinary medicine, primary food animal production, food processing and food preparation, as well as in the regulation of food safety.

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APPENDIX I: Total Aerobic Count (CFU/ml) of Milk Sample in Zaria

Location 1		Location 2	Location 3			Location 4		Location 5	
Lab No	CFU/ml 10^6	Lab No	CFU/ml 10^6	Lab No	CFU/ml 10^6	Lab No	CFU/ml 10^6	Lab No	CFU/ml 10^6
E1	3.2	E11	NG	E21	9.2	E31	1.2	E41	5.5
E2	4.6	E12	8.6	E22	8.5	E32	8.4	E42	NG
E3	4.4	E13	14.2	E23	2.8	E33	2.5	E43	2.2
E4	9.3	E14	NG	E24	NG	E34	4.6	E44	NG
E5	5.6	E15	NG	E25	4.3	E35	NG	E45	NG
E6	3.6	E16	NG	E26	NG	E36	5.7	E46	4.3
E7	7.2	E17	NG	E27	NG	E37	3.2	E47	NG
E8	2.2	E18	5.8	E28	1.9	E38	NG	E48	2.8
E9	6.6	E19	17.2	E29	NG	E39	1.1	E49	NG
E10	8.2	E20	9.8	E30	NG	E40	NG	E50	NG
B1	6.6	B11	NG	B21	7.8	B31	NG	B41	1.6
B2	8.5	B12	16.2	B22	9.6	B32	9.0	B42	1.4
B3	4.6	B13	NG	B23	5.9	B33	1.7	B43	NG
B4	2.2	B14	NG	B24	3.8	B34	NG	B44	NG
B5	1.4	B15	9.2	B25	12.3	B35	NG	B45	1.6
B6	7.8	B16	NG	B26	NG	B36	1.2	B46	NG
B7	1.2	B17	NG	B27	4.5	B37	NG	B47	NG
B8	5.3	B18	NG	B28	NG	B38	NG	B48	NG
B9	2.2	B19	NG	B29	12.9	B39	NG	B49	NG
B10	4.5	B20	2.4	B30	7.2	B40	1.9	B50	2.2
Z1	NG	Z11	NG	Z21	NG	Z31	NG	Z41	2.0
Z2	5.2	Z12	NG	Z22	1.8	Z32	2.1	Z42	NG
Z3	3.1	Z13	1.2	Z23	2.5	Z33	8.0	Z43	3.0
Z4	7.0	Z14	NG	Z24	3.4	Z34	NG	Z44	5.0
Z5	1.1	Z15	NG	Z25	NG	Z35	1.7	Z45	NG
Z6	2.5	Z16	NG	Z26	NG	Z36	NG	Z46	NG
Z7	NG	Z17	7.0	Z27	2.6	Z37	NG	Z47	NG
Z8	2.3	Z18	NG	Z28	NG	Z38	NG	Z48	NG
Z9	1.6	Z19	NG	Z29	NG	Z39	NG	Z49	NG
Z10	NG	Z20	NG	Z30	NG	Z40	NG	Z50	NG
S1	19.2	S11	6.3	S21	12.2	S31	7.4	S41	12.2
S2	NG	S12	13.2	S22	14.8	S32	11.5	S42	12.5
S3	8.0	S13	4.2	S23	13.6	S33	6.3	S43	19.4
S4	11.5	S14	NG	S24	7.2	S34	12.3	S44	8.9
S5	17.8	S15	3.1	S25	12.8	S35	13.4	S45	12.7
S6	9.0	S16	3.2	S26	8.7	S36	16.8	S46	9.2
S7	10.5	S17	14.6	S27	NG	S37	9.8	S47	13.4

	S8	11.0	S18	7.6	S28	7.8	S38	7.4	S48	4.4			
	S9	17.2	S19	9.8	S29	NG	S39	11.8	S49	7.8			
	S10	12.6	S20	5.7	S30	NG	S40	12.8	S50	9.4			

IND	MR	VP	CIT	UREA	TSI	LACT	MAN	GLU	SUC	OX	CAT	COA	ISOLATES
+	+	_	_	_	A/AG	+	+	+	+	NT	NT	NT	*E. coli*
+	+	_	_	+	A/AG	_	_	+	+	NT	NT	NT	*Proteus spp.*
_	+	_	_	_	K/A	_	+	_	_	NT	NT	NT	*Salmonella spp.*
+	+	_	+	_	K/AG	_	_	+	_	NT	NT	NT	*Providencia spp*

NG = No Growth, CFU = Colony Forming Unit

Appendix II: Typical Biochemical Reactions of Bacterial Species from Packaged Milk Sample in Zaria

_	_	+	+	_	K/AG	+	+	+	+	NT	NT	NT	*Enterobacter spp.*
_	+	_	+	+	K/AG	_	+	+	_	NT	NT	NT	*Citrobacter spp*.
_	_	+	+	+	A/AG	+	+	+	+	NT	NT	NT	*Klebsiella spp.*
_	+	_	_	+	A/A	_	+	+	+	NT	NT	NT	*Yersinia spp*.
_	_	_	+	+	K/K	_	_	_	_	+	+	NT	*Pseudomonas spp*
_	+	+	+	+	A/A	+	+	+	+	_	+	+	*Staph spp*

KEY: IND=Indole, MR=Methyl-red, VR=Voges-Proskauer, CIT=Citrate, UREA=Urease, TSI=Triple sugar iron, LACT=Lactose, MAN= Mannitol, GLU=Glucose, SUC= Sucrose, OX= Oxidase, CAT=Catalase, COA=Coagulase, A=Acid, K= Alkaline, G=Gas, NT= Not tested, + =positive, - = Negative.

APPENDIX III: Zone of Inhibition of Test Antibiotics against Bacterial Isolates

LAB NO	AMX	COT	NIT	GEN	NAL	OFL	AMC	TET	CXM	ERY	CHL	CXC
E1m	0.0±0.0	24±0.0	29±0.0	0.0±0.0	0.0±0.0	17±1.0	0.0±0.0	11±1.0	0.0±0.0	13±1.0	9±1.4	0.0±0.0
E2m	14.0±0.0	19±2.2	27±0.0	21±1.0	13±0.0	28±4.2	21±1.0	21±1.0	30±2.2	0.0±0.0	27±1.4	0.0±0.0
S5m	18.0±5.0	21±1.4	27±1.0	20±0.0	18±2.2	29±1.4	20±1.0	11±1.0	26±2.2	14±0.0	26±1.4	0.0±0.0
E43m	0.0±0.0	24±1.4	30±1.0	0.0±0.0	0.0±0.0	23±3.6	10±0.0	12±1.4	0.0±0.0	15±0.0	10±0.0	0.0±0.0
Z22m	0.0±0.0	25±1.0	0.0±0.0	0.0±0.0	19±0.0	0.0±0.0	0.0±0.0	11±0.0	0.0±0.0	14±0.0	10±0.0	0.0±0.0
S23e	0.0±0.0	0.0±0.0	14±2.8	19±1.0	22±0.0	26±6.0	0.0±0.0	0.0±0.0	18±1.0	0.0±0.0	16±2.2	0.0±0.0
E6c	0.0±0.0	0.0±0.0	0.0±0.0	14±1.0	9±0.0	27±4.0	0.0±0.0	10±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E31c	0.0±0.0	0.0±0.0	0.0±0.0	14±2.2	0.0±0.0	28±1.0	0.0±0.0	9±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E6s	0.0±0.0	0.0±0.0	0.0±0.0	16±2.8	0.0±0.0	34±2.2	0.0±0.0	10±2.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E37e	0.0±0.0	0.0±0.0	0.0±0.0	13±2.8	0.0±0.0	39±19	0.0±0.0	8±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S4c	0.0±0.0	0.0±0.0	0.0±0.0	15±2.8	0.0±0.0	24±4.2	0.0±0.0	6±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B15e	0.0±0.0	19±3.6	0.0±0.0	0.0±0.0	22±3.9	26±0.0	0.0±0.0	8±1.0	14±0.0	0.0±0.0	27±3.6	0.0±0.0
Z32s	14±2.2	27±1.0	0.0±0.0	19±2.2	20±1.0	30±0.0	22±2.3	25±1.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S1c	0.0±0.0	0.0±0.0	0.0±0.0	16±1.0	0.0±0.0	28±1.4	0.0±0.0	9±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S7c	0.0±0.0	0.0±0.0	0.0±0.0	13±1.4	0.0±0.0	22±1.0	0.0±0.0	8±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E4c	0.0±0.0	0.0±0.0	0.0±0.0	13±2.2	0.0±0.0	27±1.4	0.0±0.0	9±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B12e	0.0±0.0	17±2.2	0.0±0.0	24±1.0	12±1.0	29±4.2	0.0±0.0	11±0.0	9±1.0	0.0±0.0	0.0±0.0	0.0±0.0
E23s	10±0.0	21±1.0	0.0±0.0	13±1.0	16±6.4	27±2.2	11±1.0	15±1.0	10±0.0	0.0±0.0	10±1.0	0.0±0.0
E8c	0.0±0.0	0.0±0.0	0.0±0.0	16±3.6	0.0±0.0	29±5.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S3c	0.0±0.0	0.0±0.0	0.0±0.0	14±2.8	0.0±0.0	27±2.8	0.0±0.0	7±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E3c	0.0±0.0	0.0±0.0	0.0±0.0	14±1.0	0.0±0.0	28±1.0	0.0±0.0	8±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S21e	10±0.0	0.0±0.0	7±1.0	16±1.0	20±1.0	26±1.0	10±1.0	8±0.0	12±1.0	0.0±0.0	0.0±0.0	0.0±0.0
Z13c	0.0±0.0	16±1.0	8±1.0	24±0.0	11±0.0	26±6.4	0.0±0.0	13±2.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E1c	0.0±0.0	0.0±0.0	0.0±0.0	15±1.0	0.0±0.0	26±3.6	0.0±0.0	8±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S26e	0.0±0.0	0.0±0.0	8±1.0	16±2.2	23±1.0	29±1.0	0.0±0.0	0.0±0.0	16±1.0	0.0±0.0	0.0±0.0	0.0±0.0
E48c	0.0±0.0	0.0±0.0	0.0±0.0	16±2.2	8±0.0	28±1.0	0.0±0.0	9±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S12c	0.0±0.0	0.0±0.0	0.0±0.0	15±1.0	0.0±0.0	28±1.0	0.0±0.0	9±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Z35c	0.0±0.0	0.0±0.0	0.0±0.0	15±2.2	11±0.0	26±3.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E21s	0.0±0.0	0.0±0.0	0.0±0.0	15±1.0	0.0±0.0	31±2.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E10c	0.0±0.0	0.0±0.0	0.0±0.0	15±1.0	0.0±0.0	30±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E5c	0.0±0.0	0.0±0.0	0.0±0.0	15±1.0	0.0±0.0	29±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S5c	0.0±0.0	0.0±0.0	0.0±0.0	14±2.2	0.0±0.0	27±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S9c	0.0±0.0	0.0±0.0	0.0±0.0	15±1.0	0.0±0.0	29±2.2	0.0±0.0	9±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E25s	0.0±0.0	0.0±0.0	17±2.2	16±2.2	19±1.0	27±2.2	0.0±0.0	0.0±0.0	20±1.0	0.0±0.0	0.0±0.0	0.0±0.0
Z2s	0.0±0.0	0.0±0.0	28±2.2	19±2.2	28±3.6	30±1.0	14±1.0	14±1.0	25±5.7	0.0±0.0	23±1.0	0.0±0.0
E6e	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	26±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S39s	0.0±0.0	24±2.2	22±3.6	19±0.0	23±2.2	32±1.0	20±1.4	13±1.0	18±1.4	0.0±0.0	18±3.6	0.0±0.0
S17e	0.0±0.0	17±3.6	13±2.2	13±2.2	21±0.0	29±2.2	0.0±0.0	14±2.2	12±2.2	0.0±0.0	18±3.6	0.0±0.0
E10e	0.0±0.0	0.0±0.0	0.0±0.0	17±2.8	0.0±0.0	27±2.8	0.0±0.0	10±2.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S15e	0.0±0.0	0.0±0.0	12±1.0	21±1.4	27±2.2	30±1.0	11±1.0	10±1.0	10±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Z2e	0.0±0.0	22±1.0	0.0±0.0	17±2.4	22±2.8	29±1.4	11±0.0	20±2.2	17±5.0	0.0±0.0	17±1.0	0.0±0.0
E8s	0.0±0.0	0.0±0.0	0.0±0.0	19±2.2	0.0±0.0	32±2.8	0.0±0.0	12±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E5s	0.0±0.0	0.0±0.0	0.0±0.0	16±1.4	0.0±0.0	29±1.0	0.0±0.0	10±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E4s	0.0±0.0	0.0±0.0	0.0±0.0	13±1.4	0.0±0.0	29±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S5e	0.0±0.0	0.0±0.0	0.0±0.0	15±0.0	0.0±0.0	29±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S32e	0.0±0.0	0.0±0.0	18±0.0	17±3.6	21±2.2	29±3.6	0.0±0.0	0.0±0.0	18±2.2	0.0±0.0	0.0±0.0	0.0±0.0
B50e	17±4.2	0.0±0.0	23±1.0	19±2.2	22±1.4	33±1.0	21±1.0	16±1.4	19±0.0	0.0±0.0	23±3.6	0.0±0.0
S4e	0.0±0.0	0.0±0.0	0.0±0.0	15±1.4	0.0±0.0	31±2.2	0.0±0.0	9±1.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E39e	0.0±0.0	9±1.0	15±1.4	17±2.2	21±1.0	26±1.4	0.0±0.0	0.0±0.0	15±1.0	0.0±0.0	17±0.0	0.0±0.0
S38e	0.0±0.0	21±2.2	11±1.0	15±1.0	20±1.0	25±0.0	0.0±0.0	10±1.0	15±1.0	0.0±0.0	17±0.0	0.0±0.0
S8e	0.0±0.0	0.0±0.0	0.0±0.0	10±0.0	0.0±0.0	25±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E46e	0.0±0.0	0.0±0.0	17±1.4	13±1.0	0.0±0.0	26±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Z24s	22±1.0	0.0±0.0	26±0.0	19±0.0	27±2.2	28±1.4	23±3.6	21±1.0	15±1.0	0.0±0.0	22±1.4	0.0±0.0
E34e	17±1.4	10±1.0	17±1.4	18±0.0	21±1.0	29±1.0	29±1.0	10±1.0	16±2.2	0.0±0.0	10±1.0	0.0±0.0
S46e	0.0±0.0	0.0±0.0	18±1.4	19±1.0	27±3.6	30±1.0	0.0±0.0	0.0±0.0	18±1.0	0.0±0.0	0.0±0.0	0.0±0.0
S48e	0.0±0.0	0.0±0.0	11±1.0	17±1.4	22±1.0	30±0.0	0.0±0.0	0.0±0.0	14±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E28s	0.0±0.0	0.0±0.0	15±2.2	16±1.0	20±4.2	26±1.0	0.0±0.0	12±1.0	16±1.4	0.0±0.0	22±4.2	0.0±0.0
B7e	0.0±0.0	0.0±0.0	0.0±0.0	15±2.2	16±2.2	23±2.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B6s	0.0±0.0	0.0±0.0	0.0±0.0	13±2.2	19±1.0	24±2.2	0.0±0.0	15±1.0	0.0±0.0	0.0±0.0	12±0.0	0.0±0.0
E22s	0.0±0.0	0.0±0.0	0.0±0.0	16±1.0	0.0±0.0	19±3.6	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S31e	0.0±0.0	0.0±0.0	18±2.2	19±1.0	22±1.0	29±1.0	0.0±0.0	0.0±0.0	16±1.0	0.0±0.0	0.0±0.0	0.0±0.0
B5s	0.0±0.0	22±1.0	17±2.2	15±1.0	21±1.4	26±1.0	0.0±0.0	14±1.0	15±0.0	0.0±0.0	22±1.4	0.0±0.0
B2e	0.0±0.0	0.0±0.0	10±1.0	16±5.0	26±3.6	30±0.0	15±3.8	0.0±0.0	14±1.0	0.0±0.0	0.0±0.0	0.0±0.0
B36s	0.0±0.0	0.0±0.0	0.0±0.0	15±3.6	21±1.0	27±2.8	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B7e	0.0±0.0	0.0±0.0	13±1.0	18±2.2	0.0±0.0	26±1.4	15±1.0	0.0±0.0	0.0±0.0	0.0±0.0	19±2.2	0.0±0.0

S25e	0.0±0.0	0.0±0.0	0.0±0.0	15±1.0	0.0±0.0	25±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S47e	0.0±0.0	0.0±0.0	18±0.0	17±1.0	23±1.0	31±1.0	0.0±0.0	0.0±0.0	16±1.0	0.0±0.0	0.0±0.0	0.0±0.0
S48e	13±1.0	24±1.4	13±1.0	15±6.0	22±0.0	28±0.0	13±0.0	14±1.0	15±0.0	0.0±0.0	13±0.0	0.0±0.0
S44s	0.0±0.0	0.0±0.0	12±2.2	17±1.4	21±1.0	26±1.4	0.0±0.0	11±1.0	15±1.0	0.0±0.0	11±0.0	0.0±0.0
S35s	0.0±0.0	0.0±0.0	19±0.0	17±2.8	23±1.0	29±2.2	0.0±0.0	12±1.0	17±1.0	0.0±0.0	12±1.0	0.0±0.0
S49e	12±1.0	23±0.0	12±1.0	16±2.8	20±1.4	27±1.0	12±0.0	11±1.4	15±1.4	0.0±0.0	14±1.0	0.0±0.0
Z27s	13±0.0	26±1.0	13±1.0	15±1.0	22±1.0	29±1.0	14±2.2	13±1.0	16±1.0	0.0±0.0	11±1.0	0.0±0.0
S50s	0.0±0.0	26±0.0	15±1.0	18±1.4	22±1.0	28±2.8	11±1.0	13±1.0	19±1.4	0.0±0.0	15±1.4	0.0±0.0
S43s	0.0±0.0	0.0±0.0	0.0±0.0	16±2.2	0.0±0.0	28±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S32s	0.0±0.0	22±1.4	12±0.0	16±1.4	23±1.0	26±1.4	0.0±0.0	13±0.0	15±1.0	0.0±0.0	10±1.4	0.0±0.0
E23e	0.0±0.0	0.0±0.0	0.0±0.0	13±0.0	0.0±0.0	19±1.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S37s	0.0±0.0	0.0±0.0	13±1.4	17±1.4	21±1.4	29±1.0	0.0±0.0	0.0±0.0	17±1.4	0.0±0.0	0.0±0.0	0.0±0.0
Z23e	17±1.4	25±2.2	24±1.4	17±2.2	24±2.2	32±1.0	18±2.2	13±1.0	17±1.0	0.0±0.0	12±1.0	0.0±0.0
B3e	19±3.6	0.0±0.0	0.0±0.0	20±2.8	25±7.1	36±1.4	25±0.0	28±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B29e	20±1.4	23±1.0	20±1.4	18±2.2	23±0.0	29±1.0	20±0.0	17±2.2	19±1.0	0.0±0.0	17±1.4	0.0±0.0
B8e	0.0±0.0	20±2.2	13±1.0	16±4.2	22±2.8	28±1.0	0.0±0.0	10±0.0	18±0.0	0.0±0.0	14±1.0	0.0±0.0
B24s	0.0±0.0	0.0±0.0	21±1.4	19±1.4	23±1.4	23±1.4	30±0.0	0.0±0.0	14±1.0	19±1.0	0.0±0.0	0.0±0.0
B33e	0.0±0.0	0.0±0.0	0.0±0.0	18±2.2	0.0±0.0	21±0.0	0.0±0.0	10±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S47s	17±2.2	18±2.8	17±2.2	16±1.4	20±1.4	25±0.0	16±1.0	13±2.2	15±0.0	0.0±0.0	11±0.0	0.0±0.0
S43e	0.0±0.0	24±1.0	14±2.8	16±1.0	22±2.8	27±1.0	0.0±0.0	12±1.0	17±0.0	0.0±0.0	10±0.0	0.0±0.0
E33s	0.0±0.0	22±0.0	11±1.0	14±0.0	19±1.0	25±1.0	0.0±0.0	12±0.0	16±1.0	0.0±0.0	10±1.0	0.0±0.0
S40e	0.0±0.0	21±0.0	14±1.0	18±1.4	21±1.0	30±1.0	0.0±0.0	0.0±0.0	18±1.0	0.0±0.0	11±1.0	0.0±0.0
S39e	0.0±0.0	24±1.0	17±2.8	24±6.4	27±1.0	33±1.4	12±0.0	16±2.2	20±1.0	0.0±0.0	15±1.0	0.0±0.0
Z9s	0.0±0.0	19±1.4	15±0.0	14±1.0	19±1.0	24±3.6	0.0±0.0	10±0.0	13±1.0	0.0±0.0	11±0.0	0.0±0.0
B1e	0.0±0.0	0.0±0.0	11±1.0	14±1.4	22±1.0	29±2.8	0.0±0.0	0.0±0.0	15±0.0	0.0±0.0	10±1.0	0.0±0.0
B23s	0.0±0.0	0.0±0.0	20±1.0	18±1.4	24±1.0	31±0.0	0.0±0.0	0.0±0.0	17±1.4	0.0±0.0	11±1.0	0.0±0.0
B5s	0.0±0.0	0.0±0.0	0.0±0.0	17±2.2	0.0±0.0	21±1.0	0.0±0.0	13±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S36s	0.0±0.0	0.0±0.0	10±0.0	17±1.4	22±1.4	30±1.0	0.0±0.0	0.0±0.0	16±1.4	0.0±0.0	0.0±0.0	0.0±0.0
B23e	0.0±0.0	0.0±0.0	11±1.4	14±1.4	21±1.0	30±0.0	0.0±0.0	0.0±0.0	13v2.8	0.0±0.0	0.0±0.0	0.0±0.0
B40e	0.0±0.0	0.0±0.0	20±1.0	18±2.8	23±4.2	32±2.2	0.0±0.0	0.0±0.0	19±2.8	0.0±0.0	10±0.0	0.0±0.0
E36e	0.0±0.0	20±1.0	18±1.0	16±1.4	22±1.4	24±4.2	0.0±0.0	13±2.2	19±1.4	0.0±0.0	11±1.0	0.0±0.0
S50e	12±1.0	26±1.0	20±0.0	18±1.4	25±0.0	30±0.0	12±1.4	13±1.0	19±2.2	0.0±0.0	22±1.0	0.0±0.0
S45s	0.0±0.0	0.0±0.0	11±1.0	16±1.4	23±0.0	29±1.0	0.0±0.0	0.0±0.0	17±1.0	0.0±0.0	11±1.0	0.0±0.0
S7s	25±2.2	27±2.2	16±1.4	20±1.0	22±1.4	33±3.6	20±0.0	0.0±0.0	22±2.8	10±0.0	0.0±0.0	10±01.0
S35e	0.0±0.0	0.0±0.0	19±2.2	16±5.0	21±1.4	25±4.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B27s	0.0±0.0	0.0±0.0	0.0±0.0	19±1.0	13±1.0	28±3.6	0.0±0.0	0.0±0.0	13±3.6	0.0±0.0	0.0±0.0	0.0±0.0
Z17s	0.0±0.0	0.0±0.0	18±1.4	20±1.0	22±1.4	28±3.6	0.0±0.0	0.0±0.0	14±1.0	0.0±0.0	11±1.4	0.0±0.0
S33s	0.0±0.0	23±1.0	18±1.0	18±1.4	25±2.2	31±1.0	14±2.2	15±0.0	11±1.4	0.0±0.0	0.0±0.0	0.0±0.0
B42s	11±2.2	0.0±0.0	0.0±0.0	19±1.4	14±2.4	25±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S41e	20±0.0	23±2.2	17±1.4	16±1.0	23±1.4	28±1.0	19±1.4	16±2.2	10±1.4	0.0±0.0	18±1.0	0.0±0.0
B21e	21±3.6	24±0.0	0.0±0.0	19±1.0	27±1.4	26±1.0	23±2.2	18±2.2	18±1.0	0.0±0.0	0.0±0.0	0.0±0.0
E22e	0.0±0.0	0.0±0.0	0.0±0.0	19±0.0	0.0±0.0	14±1.0	0.0±0.0	19±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B25s	0.0±0.0	0.0±0.0	14±1.0	19±2.2	21±1.4	27±2.2	0.0±0.0	0.0±0.0	16±1.0	0.0±0.0	10±0.0	0.0±0.0
B22s	15±2.2	15±1.4	20±1.0	18±1.0	21±0.0	29±1.0	15±2.8	18±2.7	17±1.4	0.0±0.0	16±1.0	0.0±0.0
S40s	0.0±0.0	22±1.0	0.0±0.0	22±2.8	22±2.2	28±1.4	19±1.4	13±4.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B25e	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	22±2.8	25±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S34s	22±1.0	26±1.0	12±1.4	19±1.4	22±1.4	30±1.0	0.0±0.0	18±2.8	15±1.0	0.0±0.0	13±1.4	0.0±0.0
B9s	0.0±0.0	0.0±0.0	0.0±0.0	15±1.0	0.0±0.0	19±1.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B41s	22±1.0	21±1.0	18±1.4	21±4.2	23±1.4	28±3.6	22±2.8	20±0.0	18±1.4	0.0±0.0	11±1.0	0.0±0.0
S36s	0.0±0.0	0.0±0.0	0.0±0.0	17±2.2	0.0±0.0	21±1.0	0.0±0.0	11±1.0	17±1.4	0.0±0.0	0.0±0.0	0.0±0.0
B32e	0.0±0.0	0.0±0.0	14±1.4	20±0.0	23±1.0	25±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B22e	23±1.0	20±1.0	16±2.2	15±1.0	17±6.3	25±0.0	17±6.7	12±1.4	23±2.2	0.0±0.0	12±1.4	0.0±0.0
S42e	24±1.0	23±0.0	23±1.0	23±4.2	25±1.0	30±0.0	25±5.0	14±1.0	22±0.0	0.0±0.0	12±1.4	0.0±0.0
S31s	0.0±0.0	0.0±0.0	14±1.4	18±1.0	20±1.0	28±1.4	0.0±0.0	13±1.0	10±0.0	0.0±0.0	11±1.4	0.0±0.0
B45e	21±1.4	20±0.0	20±0.0	16±1.0	24±1.0	26±1.4	21±1.4	16±1.4	16±1.0	0.0±0.0	13±1.0	0.0±0.0
B10s	23±2.2	20±2.8	17±5.0	19±2.2	21±2.2	28±1.0	27±1.4	20±1.0	26±2.2	0.0±0.0	11±0.0	0.0±0.0
B27e	24±0.0	22±2.2	20±0.0	23±7.0	22±1.0	33±4.2	27±5.0	18±2.8	26±1.0	0.0±0.0	11±1.4	0.0±0.0
E18e	13±1.0	17±2.7	0.0±0.0	16±1.0	13±2.2	26±1.0	11±0.0	13±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S18e	0.0±0.0	13±2.8	0.0±0.0	23±1.4	10±1.0	23±1.4	0.0±0.0	23±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E8s	0.0±0.0	0.0±0.0	0.0±0.0	17±1.4	0.0±0.0	20±2.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Z4e	28±2.2	23±1.4	0.0±0.0	20±1.0	15±0.0	28±1.0	27±1.0	24±2.2	17±3.6	0.0±0.0	0.0±0.0	0.0±0.0
E10s	0.0±0.0	0.0±0.0	0.0±0.0	16±2.2	15±6.4	20±1.0	0.0±0.0	12±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B20e	15±0.0	0.0±0.0	0.0±0.0	18±1.4	23±0.0	25±2.2	13±0.0	0.0±0.0	17±2.2	0.0±0.0	0.0±0.0	0.0±0.0
E20e	0.0±0.0	0.0±0.0	0.0±0.0	21±13	0.0±0.0	19±2.2	0.0±0.0	11±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B30s	24±1.4	21±0.0	20±0.0	20±2.2	23±1.4	22±2.2	20±1.0	15±1.0	19±1.4	0.0±0.0	12±1.0	0.0±0.0
Z33e	0.0±0.0	0.0±0.0	0.0±0.0	19±1.4	0.0±0.0	24±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

S20e	11±1.0	0.0v0.0	0.0±0.0	18±2.2	15±1.0	20±0.0	11±1.0	14±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E19e	14±1.4	0.0±0.0	0.0±0.0	17±2.2	20±1.0	23±0.0	14±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Z3e	12±1.0	0.0±0.0	0.0±0.0	18±1.4	20±0.0	24±1.0	17±2.8	0.0±0.0	15±1.0	0.0±0.0	0.0±0.0	0.0±0.0
E12e	0.0±0.0	0.0±0.0	0.0±0.0	16±2.8	10±1.0	20±1.4	0.0±0.0	10±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Z8e	23±0.0	0.0±0.0	0.0±0.0	22±1.0	15±2.8	25±2.2	20±1.0	23±1.0	19±1.4	0.0±0.0	0.0±0.0	0.0±0.0
S10c	0.0±0.0	0.0±0.0	0.0±0.0	14v1.0	11±2.4	15±0.0	0.0±0.0	10±1.4	14±1.4	0.0±0.0	0.0±0.0	0.0±0.0
Z5e	22±1.4	28±2.2	0.0±0.0	22±1.0	22±2.2	28±3.6	23±2.3	25±2.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S44e	0.0±0.0	0.0±0.0	12±1.0	18±0.0	22±1.0	20±1.0	9.0±1.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S16c	9.0±1.4	14±1.4	0.0±0.0	25±2.2	18±3.6	28±1.4	9±0.0	13±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B4e	10±1.4	0.0±0.0	0.0±0.0	21±1.0	21±1.0	3.0±7.1	0.0±0.0	23±1.4	13±1.4	0.0±0.0	0.0±0.0	0.0±0.0
Z6e	22±1.0	16±6.4	0.0±0.0	19±2.2	0.0±0.0	27±2.2	24±2.2	20±2.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S19c	0.0±0.0	12±1.4	0.0±0.0	19±1.4	11±0.0	17±3.6	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S22e	0.0±0.0	0.0±0.0	0.0±0.0	16±2.2	8±1.0	24±1.0	0.0±0.0	11±1.0	9±1.0	0.0±0.0	0.0±0.0	0.0±0.0
S6c	0.0±0.0	0.0±0.0	0.0±0.0	17±2.2	10±0.0	21±1.0	0.0±0.0	11±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E13e	22±1.4	21±1.4	20±1.0	17±1.4	19±1.4	26±1.4	21±1.4	13±1.0	16±2.2	0.0±0.0	10±0.0	0.0±0.0
E32s	13±1.0	0.0±0.0	0.0±0.0	18±2.8	0.0±0.0	0.0±0.0	11±1.0	12±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E4e	0.0±0.0	0.0±0.0	0.0±0.0	15±1.0	0.0±0.0	17±2.2	0.0±0.0	11±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S13c	0.0±0.0	15±1.0	0.0±0.0	23±1.0	11±1.4	25±4.2	0.0±0.0	13±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S24e	0.0±0.0	0.0±0.0	0.0±0.0	15±1.0	10±1.4	18±1.0	0.0±0.0	12±1.0	10±1.0	0.0±0.0	0.0±0.0	0.0±0.0
E41c	0.0±0.0	0.0±0.0	0.0±0.0	17±1.4	10±0.0	19±1.0	0.0±0.0	11±1.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S19e	0.0±0.0	13±4.2	0.0±0.0	21±2.2	11±1.4	24±1.0	0.0±0.0	11±1.0	9±1.0	0.0±0.0	0.0±0.0	0.0±0.0
E7e	0.0±0.0	0.0±0.0	0.0±0.0	17±1.4	10±1.0	21±1.0	0.0±0.0	11±1.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Antibiotics	Abbreviation
Amoxicillin	AMX
Cotrimoxazole	COT
Nitrofurantoin	NIT
Gentamicin	GEN
Nalidixic acid	NAL
Ofloxacin	OFL
Amoxicillin/clavulanic acid	AMC
Tetracycline	TET
Cefuroxime	CXM
Erythromycin	ERY
Chloramphenicol	CHL
Cloxacillin	CXC

APPENDIX IV: Antibiotics Susceptibility Pattern of Bacterial Isolates

LAB NO	ISOLATE	AMX	COT	NIT	GEN	NAL	OFL	AMC	TET	CXM	ERY	CHL	CXC
S5e	*E. coli*	R	R	R	S	R	S	R	R	R	R	R	R
S32e	*E. coli*	R	R	S	S	S	S	R	R	S	R	R	R
B50e	*E. coli*	S	R	S	S	S	S	S	I	S	R	I	R
S4e	*E. coli*	R	R	R	S	R	S	R	R	R	R	R	R
E39e	*E. coli*	R	R	I	S	S	S	R	R	I	R	I	R
S38e	*E. coli*	R	S	R	S	S	S	R	R	I	R	I	R
S8e	*E. coli*	R	R	R	R	R	S	R	R	R	R	R	R
E46e	*E. coli*	R	R	S	I	R	S	R	R	R	R	R	R
Z24s	*E. coli*	S	R	S	S	S	S	S	S	I	R	S	R
E34e	*Klebsiella spp*	S	R	S	S	S	S	R	R	I	R	R	R
S46e	*Klebsiella spp*	R	R	S	S	S	S	R	R	S	R	R	R
E25s	*Citrobacter spp*	R	R	S	S	S	S	R	R	S	R	R	R
Z2s	*Yersinia spp*	R	R	S	S	S	S	I	R	S	R	S	R
E6e	*Providencia spp*	R	R	R	R	R	S	R	R	R	R	R	R
S39s	*Providencia spp*	R	S	S	S	S	S	S	S	S	R	S	R
S17e	*Providencia spp*	R	S	R	I	S	S	R	R	R	R	S	R
E10e	*Providencia spp*	R	R	R	S	R	S	R	R	R	R	R	R
S15e	*Providencia spp*	R	R	R	S	S	S	R	R	R	R	R	R
Z2e	*Proteus spp*	R	S	R	S	S	S	R	R	R	R	R	R
E8s	*Proteus spp*	R	R	R	S	R	S	R	R	R	R	R	R
E5s	*Proteus spp*	R	R	R	S	R	S	R	R	R	R	R	R
E4s	*Proteus spp*	R	R	R	I	R	S	R	R	R	R	R	R
S48e	*Enterobacter spp*	R	R	R	S	S	S	R	R	R	R	R	R
E28s	*Enterobacter spp*	R	R	I	S	S	S	R	R	I	R	S	R
B7e	*Enterobacter spp*	R	R	R	S	I	S	R	R	R	R	R	R
B6s	*Enterobacter spp*	R	R	R	I	S	S	R	I	R	R	R	R
E22s	*Enterobacter spp*	R	R	R	S	R	S	R	R	R	R	R	R
S31e	*Enterobacter spp*	R	R	S	S	S	S	R	R	I	R	R	R
B5s	*Enterobacter spp*	R	R	S	S	S	S	R	R	I	R	R	R
B2e	*Enterobacter spp*	R	R	R	S	S	S	I	R	R	R	R	R
B36s	*Enterobacter spp*	R	R	R	S	S	S	R	R	R	R	R	R
B7s	*Enterobacter spp*	R	R	R	S	R	S	I	R	R	R	S	R
S47e	*E. coli*	R	R	S	S	S	S	R	R	I	R	R	R
S48s	*E. coli*	R	S	R	S	S	S	R	R	I	R	I	R
S44s	*E. coli*	R	R	R	S	S	S	R	R	I	R	R	R
S35s	*E. coli*	R	R	S	S	S	S	R	R	I	R	R	R
S49e	*E. coli*	R	S	R	S	S	S	R	R	I	R	R	R
Z27s	*E. coli*	R	S	R	S	S	S	I	R	I	R	I	R
S50s	*E. coli*	R	S	I	S	S	S	R	R	S	R	R	R
S43s	*E. coli*	R	R	R	S	R	S	R	R	R	R	I	R
S32s	*E. coli*	R	S	R	S	S	S	R	R	I	R	R	R
E23e	*E. coli*	R	R	R	I	R	S	R	R	R	R	R	R
S37s	*E. coli*	R	R	R	S	S	S	R	R	I	R	R	R
Z23e	*Enterobacter spp*	S	S	S	S	S	S	S	R	I	R	R	R
B3e	*Enterobacter spp*	S	R	R	S	S	S	S	S	R	R	R	R
B29e	*Enterobacter spp*	S	S	S	S	S	S	S	I	S	R	I	R
B8e	*Enterobacter spp*	R	S	R	S	S	S	R	R	S	R	I	R

B24s	*Enterobacter spp*	R	R	S	S	S	S	R	R	S	R	R	R
B33e	*Enterobacter spp*	R	R	R	S	R	S	R	R	R	R	R	R
S47s	*Enterobacter spp*	S	S	S	S	S	S	I	R	I	R	R	R
S43e	*Enterobacter spp*	R	S	R	S	S	S	R	R	I	R	R	R
E33s	*Enterobacter spp*	R	S	R	I	S	S	R	R	I	R	R	R
S40e	*Enterobacter spp*	R	S	R	S	S	S	R	R	S	R	R	R
S39e	*Enterobacter spp*	R	S	S	S	S	S	R	I	S	R	I	R
Z9s	*Enterobacter spp*	R	S	I	I	S	S	R	R	R	R	R	R
B1e	*Enterobacter spp*	R	R	R	I	S	S	R	R	I	R	R	R
B23s	*Enterobacter spp*	R	R	S	S	S	S	R	R	I	R	R	R
B5s	*Enterobacter spp*	R	R	R	S	R	S	R	R	R	R	R	R
S36e	*Enterobacter spp*	R	R	R	S	S	S	R	R	I	R	R	R
B23e	*Enterobacter spp*	R	R	R	R	S	S	R	R	R	R	R	R
B40e	*Enterobacter spp*	R	R	S	S	S	S	R	R	S	R	R	R
E36e	*Enterobacter spp*	R	S	S	S	S	S	R	R	S	R	R	R
S50e	*Enterobacter spp*	R	S	S	S	S	S	R	R	S	R	S	R
S45s	*Enterobacter spp*	R	R	R	S	S	S	R	R	I	R	R	R
S7s	*Salmonella spp*	S	S	I	S	S	S	S	R	S	R	R	R
S35e	*Enterobacter spp*	R	R	S	S	S	S	R	R	R	R	R	R
B27s	*Enterobacter spp*	R	R	R	S	R	S	R	R	R	R	R	R
Z17s	*Enterobacter spp*	R	R	S	S	S	S	R	R	R	R	R	R
S33s	*Enterobacter spp*	R	S	S	S	S	S	I	I	R	R	R	R
B42s	*Enterobacter spp*	R	R	R	S	I	S	R	R	R	R	R	R
S41e	*Enterobacter spp*	S	S	S	S	S	S	S	I	R	R	S	R
B21e	*Enterobacter spp*	S	S	R	S	S	S	S	I	S	R	R	R
E22e	*Enterobacter spp*	R	R	R	S	R	I	R	S	R	R	R	R
B25s	*Enterobacter spp*	R	R	R	S	S	S	R	R	I	R	R	R
B22s	*Enterobacter spp*	I	I	S	S	S	S	I	I	I	R	I	R
S40s	*Enterobacter spp*	R	S	R	S	S	S	S	R	R	R	R	R
B25e	*Enterobacter spp*	R	R	R	R	S	S	R	R	R	R	R	R
S34s	*Enterobacter spp*	S	S	R	S	S	S	R	I	I	R	I	R
B9s	*Enterobacter spp*	R	R	R	S	R	S	R	R	R	R	R	R
B41s	*Enterobacter spp*	S	S	S	S	S	S	S	S	S	R	R	R
S36s	*Enterobacter spp*	R	R	R	S	R	S	R	R	I	R	R	R
B32e	*Enterobacter spp*	R	R	R	S	S	S	R	R	R	R	R	R
B22e	*Enterobacter spp*	S	S	I	S	I	S	I	R	S	R	R	R
S42e	*Enterobacter spp*	S	S	S	S	S	S	S	R	S	R	R	R
S31s	*Enterobacter spp*	R	R	R	S	S	S	R	R	R	R	R	R
B45e	*Enterobacter spp*	S	S	S	S	S	S	S	I	I	R	I	R
B10s	*Enterobacter spp*	S	S	S	S	S	S	S	S	S	R	R	R
B27e	*Enterobacter spp*	S	S	S	S	S	S	S	I	S	R	R	R
B30s	*Enterobacter spp*	S	S	S	S	S	S	S	I	S	R	R	R
S44e	*Enterobacter spp*	R	R	R	S	S	S	R	R	R	R	R	R
E1m	*Staph. aureus*	R	S	S	R	R	S	R	R	R	R	R	R
E2m	*Staph. aureus*	R	S	S	S	R	S	S	S	S	R	S	R
S5m	*Staph. aureus*	R	S	S	S	I	S	S	R	S	I	S	R
E43m	*Staph. aureus*	R	S	S	R	R	S	R	R	R	I	R	R
Z22m	*Staph. aureus*	R	S	S	R	R	S	R	R	I	I	R	R
S23e	*Pseudomonas spp*	R	R	R	S	S	S	R	R	S	R	I	R
E6c	*Pseudomonas spp*	R	R	R	I	R	S	R	R	R	R	R	R
E31c	*Pseudomonas spp*	R	R	R	I	R	S	R	R	R	R	R	R
E6s	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
E37e	*Pseudomonas spp*	R	R	R	I	R	S	R	R	R	R	R	R

S4c	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
B15e	*Pseudomonas spp*	R	S	R	R	S	S	R	R	R	R	S	R
Z32s	*Pseudomonas spp*	R	S	R	S	S	S	S	S	R	R	R	R
S1c	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
S7c	*Pseudomonas spp*	R	R	R	I	R	S	R	R	R	R	R	R
E4c	*Pseudomonas spp*	R	R	R	I	R	S	R	R	R	R	R	R
B12e	*Pseudomonas spp*	R	S	R	S	R	S	R	R	R	R	R	R
E23s	*Pseudomonas spp*	R	S	R	I	I	S	R	I	R	R	R	R
E8c	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
S3c	*Pseudomonas spp*	R	R	R	I	R	S	R	R	R	R	R	R
E3c	*Pseudomonas spp*	R	R	R	I	R	S	R	R	R	R	R	R
S21e	*Pseudomonas spp*	R	R	R	S	S	S	R	R	R	R	R	R
Z13c	*Pseudomonas spp*	R	S	R	S	R	S	R	R	R	R	R	R
E1c	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
S26e	*Pseudomonas spp*	R	R	R	S	S	S	R	R	I	R	R	R
E48c	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
S12c	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
Z35c	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
E21s	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
E10c	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
E5c	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
S5c	*Pseudomonas spp*	R	R	R	I	R	S	R	R	R	R	R	R
S9c	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
S25e	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
E18e	*Pseudomonas spp*	R	S	R	S	R	S	R	R	R	R	R	R
S18e	*Pseudomonas spp*	R	I	R	S	R	S	R	R	R	R	R	R
E8e	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
Z4e	*Pseudomonas spp*	S	S	R	S	I	S	S	S	R	R	R	R
E10s	*Pseudomonas spp*	R	R	R	S	I	S	R	R	R	R	R	R
B20e	*Pseudomonas spp*	I	R	R	S	S	S	I	R	R	R	R	R
E20e	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
Z33e	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
S20e	*Pseudomonas spp*	R	R	R	S	I	S	R	R	R	R	R	R
E19e	*Pseudomonas spp*	I	R	R	S	S	S	I	R	R	R	R	R
Z3e	*Pseudomonas spp*	R	R	R	S	S	S	S	R	R	R	R	R
E12s	*Pseudomonas spp*	R	R	R	S	R	S	R	S	S	R	R	R
Z8e	*Pseudomonas spp*	S	R	R	S	I	S	S	S	I	R	R	R
S10c	*Pseudomonas spp*	R	R	R	I	R	S	R	R	R	R	R	R
Z5e	*Pseudomonas spp*	S	S	R	S	S	S	S	S	R	R	R	R
S16c	*Pseudomonas spp*	R	I	R	S	I	S	R	R	R	R	R	R
B4e	*Pseudomonas spp*	R	R	R	S	S	S	R	S	R	R	R	R
Z6e	*Pseudomonas spp*	S	S	R	S	R	S	S	S	R	R	R	R
S19c	*Pseudomonas spp*	R	I	R	S	R	S	R	R	R	R	R	R
S22e	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
S6c	*Pseudomonas spp*	R	R	S	S	R	S	R	R	R	R	R	R
E13e	*Pseudomonas spp*	S	S	R	S	S	S	S	R	R	R	R	R
E32s	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
E4e	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
S13c	*Pseudomonas spp*	R	I	R	S	R	S	R	R	R	R	R	R
S24e	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
E41c	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R
S19e	*Pseudomonas spp*	R	I	R	S	R	S	R	R	R	R	R	R
E7e	*Pseudomonas spp*	R	R	R	S	R	S	R	R	R	R	R	R

R= RESISTANT, I = INTERMEDIATE, S= SENSITIVE

APPENDIX V: Minimum Inhibitory Concentration (MIC) µg/ml of Four Antibiotics against Twenty-six Enterobacteriaceae

S/NO	LAB NO	AMX	CHL	OFL	TET
1	S5e	1000	200	12.5	20
2	S4e	500	10	0.781	100
3	S8e	1000	50	3.125	50
4	E46e	1000	200	0.391	20
5	E6e	1000	10	3.125	50
6	E10e	1000	10	3.125	50
7	S15e	1000	10	1.563	50
8	E8s	1000	10	1.563	5
9	E5s	1000	20	6.25	5
10	E4s	1000	200	3.125	50
11	S48e	1000	100	6.26	20
12	B7e	1000	5	6.25	200
13	E22s	200	200	6.25	100
14	B36s	1000	20	1.563	200
15	S43s	1000	5	6.25	200
16	E23e	1000	5	12.5	50
17	B33e	500	5	12.5	50
18	B5s	500	200	6.25	50
19	B23e	1000	5	6.25	100
20	B27s	1000	5	1.563	50
21	E22e	1000	200	6.25	100
22	B25e	1000	10	1.563	200
23	B41s	1000	10	1.563	100
24	S36s	1000	10	1.563	100
25	S31s	1000	200	3.125	100
26	S44e	1000	10	1.563	200

MIC RANGE

AMOXICILLIN 5 - 2000µg/ml

CHLORAMPHENICOL 1.25 - 1000µg/ml

OFLOXACIN 0.1875 - 100 µg/ml

TETRACYCLINE 1.25 - 1000 µg/ml

APPENDIX VI:Standard Molecular Weight Sizes of DNA Ladder

Molecular sizes of standard DNA ladder (100bp plus)	Log of molecular sizes of ladder	Distance moved by the band in cm
3000	3.5	1.0
2000	3.3	1.2
1500	3.2	1.4
1200	3.1	1.5
1000	3.0	1.6
900	2.95	1.65
800	2.9	1.7
700	2.85	1.8
600	2.8	1.9
500	2.7	2.0
400	2.6	2.2
300	2.5	2.3
200	2.3	2.4
100	2.0	2.5

This shows the distance moved by each bands of 100bp DNA ladder. This was used as molecular weight standards for agarose gel electrophoresis.

APPENDIX VIIA: Phenotypic Resistance Pattern of Isolates from Milk Sample in Zaria.

S/N	Resistance Pattern	No. of Isolate
1	Amx, Amc, Chl, Cot, Cxc, Cxm, Ery, Gent, Nal, Nit, Tet	2
2	Amx, Amc, Chl, Cot, Cxc, Cxm, Ery, Nal, Nit, Tet	13
3	Amx, Amc, Chl, Cot, Cxc, Cxm, Ery, Gent, Nit, Tet	2
4	Amx, Amc, Chl, Cot, Cxc, Cxm, Ery, Nal, Tet	1
5	Amx, Amc, Chl, Cot, Cxc, Cxm,Ery, Nit, Tet	8
6	Amx, Amc, Chl, Cot, Cxc, Cxm, Ery, Nal, Nit	1
7	Amx, Amc, Chl, Cot, Cxc,Ery, Nal, Nit, Tet	1
8	Amx, Amc, Chl, Cot, Cxc, Cxm, Ery, Nit	1
9	Amx, Chl, Cot, Cxc, Cxm, Ery, Nit, Tet	1
10	Amx, Cot, Cxc, Cxm, Ery, Nal, Nit, Tet	1
11	Amx, Amc, Chl, Cot, Cxc, Ery, Nit, Tet	6
12	Amx, Amc, Chl, Cot, Cxc, Cxm, Ery, Tet	2
13	Amx, Amc, Chl, Cot, Cxc, Ery, Tet	9
14	Amx, Amc, Cxc, Cxm, Ery, Nit, Tet	1
15	Amx, Amc, Chl, Cxc, Ery, Nit, Tet	4
16	Amx, Amc, Chl, Cxc, Cxm, Ery, Tet	1
17	Amx, Chl, Cxc, Cxm, Ery, Nit, Tet	1
18	Amx, Amc, Cot, Cxc, Ery, Tet	2
19	Amx, Amc, Cxc, Ery, Nit, Tet	4

20	Amc, Chl, Cot, Cxc, Ery, Tet	1

APPENDIX VIIB: Phenotypic Resistance Pattern of Isolates from Milk Sample in Zaria

S/N	Resistance Pattern	No of Isolates
21	Amx, Chl, Cxc, Ery, Nit, Tet	1
22	Chl, Cot, Cxc, Cxm, Ery, Nit	1
23	Amx, Amc, Chl, Cxc, Ery, Tet	1
24	Amx, Cot, Cxc, Ery, Tet	1
25	Amx, Amc, Cxc, Ery, Nit	1
26	Amx, Amc, Chl, Ery, Tet	1
27	Amx, Amc, Cxc, Ery, Tet	2
28	Amx, Chl, Cxc, Cxm, Ery	1
29	Chl, Cxc, Ery, Tet	5
30	Amx, Amc, Cxc, Ery	1
31	Chl, Cxc, Ery, Nit	1
32	Amc, Cxc, Ery, Nit	1
33	Cot, Cxc, Ery	2
34	Amx, Cxc, Ery	1
35	Cxc, Cxm, Ery	1
36	Chl, Cxc, Ery	4
37	Cxc, Ery	3
	TOTAL	90

KEY: Amx= Amoxicillin, Amc= Amoxicillin/clavulanic acid, Chl= Chloramphenicol, Cot= Cotrimoxazole, Cxc= Cloxacillin, Cxm= Cefuroxime, Ery= Erythromycin, Gent= Gentamicin, Nal= Nalidixic acid, Nit= Nitrofurantoin, Tet= Tetracycline.