ISOLATION AND MOLECULAR CHARACTERISATION OF SOME BACTERIA FROM FRESH RAW COW MILK AND HANDLERS IN ZARIA METROPOLIS KADUNA STATE NIGERIA

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BY

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DECLARATION

I, declare that the work reported in this dissertation titled "**Isolation and Molecular Characterisation of some Bacteria from Fresh Raw Cow Milk and Handlers in Zaria Metropolis Kaduna State Nigeria"** was carried out by me in the Department of Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, under the Supervision of Prof. J.A. Onaolapo and Dr. R.O. Bolaji. The information derived from the literature review has been duly acknowledged in the text and list of the reference provided. No part of this dissertation has been presented in any previous application for another degree at any University.

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CERTIFICATION

The project entitled ISOLATION AND MOLECULAR CHARACTERISATION OF SOME BACTERIA FROM FRESH RAW COW MILK AND HANDLERS IN ZARIA METROPOLIS KADUNA STATE by Bilikis Abimbola OLUNREBI meets the regulations governing the award of Masters of Science in Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I would like to dedicate this project to Almighty Allah, the helper and sustainer of men. To Him belong all the glory and honor, power and praise forever (Alhamdulillah).

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ABSTRACT

Raw cow milk is one of the most consumed products of animal origin, it is an important component of human nutrition, especially in the period of growth. Relatively, the composition of milk makes it an optimum medium for the growth of microorganisms, making it an efficient vehicle for transmission of disease-causing agents to humans. It is therefore, optimally necessary to safe guard the quality of raw milk processed all through the dairy chain. The microbiological quality of fresh raw cow milk from four farms in Zaria metropolis, Kaduna state were evaluated. The samples were made up of 42 raw cow milks from lactating cows available, 42 swabs from cow teat; 16 swabs from herd handlers and 5 samples from water used in the cleaning process making a total of 105. The total aerobic bacteria count (TABC) and total coliform count (TCC) of raw milk were carried out using plate count method on Nutrient and MacConkey media respectively. Serial dilutions were carried out using peptone water on all the forty-two (42) raw milk samples. Isolation of *Staphylococcus species* and enteric microorganisms were carried out using plate count method on Mannitol Salt Agar and MacConkey media respectively. The isolates were identified using standard biochemical procedure and Microgen ^{TM} System. Antimicrobial Susceptibility test was carried out on the identified isolates using the modified Kirby-Bauer method. Using methods by Blanchard and Nedrud, *Helicobacter pylori* was cultured on blood enriched medium supplemented with vancomycin powder. One hundred and two (102) bacteria consisting of Seventy-six (76) Polymicrobial and twenty-six (26) single cultures were recovered as positive culture while three (3) had no growth. No significant association was observed between sampling location and positive bacterial recovery (0.270). The mean TABC and TCC of raw milk observed in this study were 2.56 ± 0.40 x10^4cfu/ml and 1.06 ± 0.16 x10^4cfu/ml respectively. *Acinetobacter iwoffi* and other members of Enterobacteriaceae isolates were resistant to tetracycline (68.75%), erythromycin (71.74%) and metronidazole (100%), while *Staphylococcus species* and *Micrococcus luteus* isolated were methicillin resistant (85%) and resistant to tetracycline was 75%. High susceptibility was observed to gentamicin (94.34%) and chloramphenicol (80.85%) by the Enterobacteriaceae isolates, while Chloramphenicol (90%) and ciprofloxacin (85%) were also active against both the *Staphylococcus species* and *M. luteus* identified in this study. Overall, about 53.8% of the isolates were multiantibiotic resistant. There was no significant association between sample source and multi-resistance phenotype, however, isolates from teat swabs were more likely to be non- multiantibiotic resistant ($p= 0.555$). No significant association between sampling location and multi-resistance phenotype was observed ($p= 0.145$). The percentage of multiple antibiotic resistance index (MARI) of greater than or equal to 0.3 was observed to be 90% for *Staphylococcus species* and 92% for Enterobacteriaceae. There was amplification of *nim* at 458bp from *Proteus mirabilis* isolated from the handler and *ermB* at 639bp from *Pseudomonas aeruginosa* and *Proteus vulgaris* from raw milk and *S. choleraesus* from the handler. Similarly, *tetA* (210bp) was obtained from *P. aeruginosa* and *P. vulgaris* all from raw milk. The amplification of *cagA* virulence marker of *H. pylori* isolated from raw milk samples establishes the possibility of the transfer to raw milk consumers via cow milk. This study reveals high contamination rates coupled with antibiotic resistance of the isolates in Zaria metropolis which poses a serious therapeutic challenge to the management of food borne acquired diseases.

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List of Abbreviations

S. aureus - *Staphylococcus aureus*

DNA- Deoxyribonucleic Acid

MARI- Multiple antibiotics Resistance Index

MDR – Multidrug Resistance

XDR- Extended Drug Resistance

PCR- Polymerase chain reaction

DR- Drug Resistant

COPs- Coagulase Positive *Staphylococcus*

National Animal Production Research Institute (NAPRI)

CHAPTER ONE

1.1 INTRODUCTION

Milk represents an important food product of animal origin. It is the most complete and easily assimilated organism foodstuff, being one of the most important especially in the period of growth, because it contains all the necessary nutrients for the normal growth and development of the organism. The most evident proof is that the newborns live and develop normally, only with milk, long after their birth (Golban, 2015). Also, milk is called the white blood or the health spring, by its high value and it is strictly necessary in the feeding of the sick persons, old people and those who work in the toxic environment. Milk helps in fighting against diseases such as gout, kidney stones, breast cancer, rheumatoid arthritis, migraine headaches, amongst others.

Fresh raw cow milk is secreted in the cow's udder- a hemispherical organ divided into right and left halves by a crease. Each half is divided into quarters by a shallower transverse crease and each quarter has one teat with its own separate mammary gland. It is therefore theoretically possible to get fresh raw cow milk of four different qualities from the same cow. The cow's udder is composed of glandular tissue containing milk producing cells. Traditional milking practices by hand has been the most practiced system since old times (Figure1.1). Mostly cows are milked by the same people every day and they become accustomed to their handler; the milk let down is stimulated by the familiar sounds of the process. The conventional milking systems involve milking machine with the use of a vacuum pump, a vacuum vessel for collecting milk, teat cup and a pulsator. The conventional milking system is advantageous in terms of ensuring proper hygiene and time management, but strict care and trained personnel are needed so as not to inflict pain on the cow (FAO 2019).

Figure 1.1: Milking of Cow

This Study*:* National Animal Production Research Institute (NAPRI**)**

The extremely nourishing nature of dairy products; neutral pH of milk and beef makes them particularly suitable media for bacterial proliferation and also, serves as vehicles for transmission of foodborne pathogens to humans (Melini *et al.,* 2017). Depending on the handling and processing it is subjected to, milk can have its physical, chemical and biological properties easily altered by the actions of microorganisms. Contaminated milk product is one of the world's most dangerous food products (CDC. 2012).

Due to its characteristics, milk deserves special attention in its production, processing, marketing and consumption. Several factors, such as the health of the animal udder, sterility of the cleaning equipment and utensils used to obtain it, the sanitary conditions of the milking place and quality of

water used on the farm, may influence the microbiological quality of milk products. The US Centers for Disease Control (CDC) says improperly handled fresh raw cow milk is responsible for nearly three times more hospitalizations than any other food-borne disease source (CDC. 2012).

1.2 Statement of Research Problem

There have been numerous outbreaks of diseases in humans with pathogens, mainly from food of animal origin and this has led to severe health consequences (WHO 2020). Milk can act as a vehicle for the transmission of bacterial diseases such as, Salmonellosis, *E. coli* infections, Cholera, Brucellosis, Streptococcal infections and Listeriosis. Also, the consumption of milk with antimicrobial residue above the Maximum Residue Limit (MRL), has a negative economic public effect on the populace, this can cause and stimulate some allergic reactions, gastrointestinal disorder, neurological disorder, cancer, antimicrobial resistance among others (Delatour *et al.,* 2018; Du *et al.,* 2019). In a report of food poisoning outbreak, 19% of the illness were caused by *Salmonella specie*, 15% of the illness by shiga toxin producing *E. coli* (0:157), the most affected people consuming raw cow milk product are the children between age one and 4 years (CDC. 2016).

Earlier studies on microbial quality of milk showed that the presence of bacterial contamination in fresh raw cow milk are mostly as a result from unhygienic practices during the pre-milking and storage stage. Samples were found contaminated with *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Raghvendra *et al.,* 2017). The biggest challenge for most of the developing countries is how to improve the dairy value chain; therefore, some great efforts have been established to provide maximum possible solutions related to the improvement of the milk quality. Reports of *Staphylococcal* food poisoning (SFP) have raised public health concerns particularly from consumption of contaminated food including raw milk and beef from infected cattle, infected food handlers and contaminated utensils or environment (Omoshaba *et al.,* 2018)

Mastitis caused by coliform is also a cause of concern. The infection is usually confined to one or two quarter of the udder and the infection occurs very fast such that a cow may appear healthy during a milking process and be sick during the next. *Escherichia coli,* a major inhabitant of the intestines of all animals is mostly associated with coliform mastitis, when this enteric organism is allowed to spread unchecked because of unhygienic practices during milking process and dirty environmental factors, these could lead to serious problem and threat to the dairy chain.

To this end, molecular based technologies, which are highly sensitive and offers high promising possibility of testing a large number of pathogenic microorganism at various points of the value chain, were developed and have shown remarkable improvement in the dairy chain processes (Feitosa *et al*., 2017).

In Nigeria, several workers have reported that milk products can be contaminated with several bacterial pathogens such as *Staphylococcus species, E. coli, Klebsiella species, Enterobacter species and Salmonella species (*Umofia *et al.,* 2014; Okpo *et al.,* 2017; Omoshaba *et al.,* 2018; Aliyu *et al.,* 2019). The presence of these bacteria in milk products poses health hazards to consumers in this area. Coliform organisms in milk have been linked to a wide variety of human infections such as endocarditis, urinary and genital tract infections, meningitis and septicemia (CDC 2015). In this regard, similar studies related to ensuring fresh raw cow milk safety need to be carried out in order to provide reliable information which can improve the quality of milk. Fresh raw cow milk contaminated 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 (Okechukwu *et al.,* 2020).

1.3 Justification

Improper cleaning and sanitizing of the cow's udder is one of the major sources of high-level bacteria count in fresh raw cow milk. Maldaner *et al., (*2012) reported that in the two properties assessed by their study, the udder cleaning process was performed incorrectly, reinforcing the need for continuous training focused on showing dairy farmer how important it is to sanitize the udders of the cows in order to have raw cow milk with low bacterial count. According to Mhone *et al.,* 2011 and Suranindyah *et al.,* 2015, the high amounts of bacteria found in refrigerated fresh raw cow milk can be explained by the fact that in small farms there are several hygiene problems occurring during milking and raw cow milk storing processes.

In most developing countries, surveillance and reporting of food-borne diseases are not adequately recorded which makes it extremely difficult to estimate and record infections relating to raw milk and milk products consumption. This makes it extremely necessary to undertake studies on the quality of packaged milk sold in different areas of the country. The multiple antibiotic resistances observed among some bacteria isolates from milk products has constitute problems in infections that account for most of Africa's disease burden, including respiratory and diarrheal diseases (CDC 2015; Du. *et al.,* 2019). Milk products have been contaminated with pathogens that are resistant to several antibiotics, and they continue to pose significant clinical threat to consumers and economic threats to the milk processing industry (Delatour *et al.,* 2018; Okechukwu *et al.,* 2020).

The hygienic quality of raw cow milk is of crucial importance in producing milk product that are safe and suitable for their intended uses. To achieve this quality, good hygienic practices should be applied throughout the dairy chain. Dairy producers especially small-scale farmers are faced with difficulties in the handling and processing practices; insufficient knowledge and skills in hygienic practices; lack of financial incentives for milk quality improvement. Milk quality control testing is

therefore necessary and should be carried out at all stages of the dairy chain. The Organoleptic characteristics, physical and chemical characteristics, quantity, hygienic standard using suitable chemical and microbial analysis should be of great importance as routine checks in other to achieve a desirable milk quality (CDC 2015). To this regard, molecular characterization of antimicrobial resistant bacteria isolates found in fresh raw cow milk samples as well as the entire milking process ranging from herd handlers, water used in cleaning, to the milking environment will be an update on the present knowledge gap of the dairy value chain.

1.4 Aim of Research

To evaluate some bacteria isolates, present in fresh raw cow milk samples and observe the bacteria susceptibility to commonly prescribed antimicrobials as well as molecularly characterize resistant bacteria isolates in selected arears around Zaria metropolis.

1.5 Specific Objectives

The Objectives of the research are to:

1. Determine the Total Aerobic Bacteria Count and identify the bacteria contaminants found in raw cow milk samples using surface plating method in suitable biochemical processes.
2. Determine the antimicrobial susceptibility pattern of the bacteria isolates to commonly prescribed antimicrobials using the modified Kirby-Bauer method.
3. Detect resistant genes (*mecA, nim, tet, ermB*) using Polymerase Chain Reaction (PCR).
4. Screen for *Helicobacter pylori* using standard cultural technique and *glmM* gene marker.
5. Detect *cagA* virulence gene from the *Helicobacter pylori* isolates, if

   Found, this will be used as a predictive marker for *Helicobacter pylori* pathogenicity.

1.6 Hypothesis

1.6.1 Null Hypothesis:

Fresh raw cow milk produced in Zaria; Nigeria is not contaminated with bacterial pathogens.

1.6.2 Alternate Hypothesis:

Fresh raw cow milk produced in Zaria; Nigeria is contaminated with bacterial pathogens.

1.7 Scope of the study

Prospective study on fresh raw cow milk "Madara" produced in Zaria. Fresh raw cow milk samples were gotten directly from four different farm locations and swabs were taken from the cow teat, handlers and some water used in cleaning processes. The farms are:

a) Zango, Sabon-Gari L.G.A

b) Jamaa Lima, Sabon-Gari L.G.A

c) Kufena, Zaria L.G.A

d) NAPRI, Shika-Giwa L.G.A

Bacteriological analysis which involved isolation, identification and antimicrobial susceptibility testing were carried out at Postgraduate Laboratory of the Department of Pharmaceutical Microbiology, Ahmadu Bello University Zaria, Kaduna State. Molecular analyses on the resistant bacteria isolates were carried out at National Biotechnology Development Agency (NABDA) in the Agricultural Biotechnology Department Abuja.

CHAPTER TWO

2.1 LITERATURE REVIEW

2.1.1 Fresh raw cow milk Quality

Milk is an aqueous colloidal suspension of protein, fats, carbohydrates and it contains numerous vitamins and minerals (Ogbolu *et al.,* 2014). Raw cow milk is milk that has not been pasteurized or homogenized (CDC 2012). Raw cow milk essentially comes from a sterile environment within the animal, the risk of contamination begins within the udder and other external contaminations by the skin, feces, milking equipment, handling and storage (John 2015).

Consumer demand for organic and natural foods (minimally processed foods) has been on the rise with great benefit attributed to these food products. However, in contrast to some perceptions, natural food products are not necessarily safer than conventional ones, foodborne illness associated with direct consumption of natural food especially unpasteurized dairy products has been a major burden in major cities around the world (Robinson *et al.,* 2014; Mungai *et al.,* 2015). Most raw cow milk consumers argue that it is a complete, natural food containing more amino acid, antimicrobials, vitamins, minerals and fatty acids than pasteurized milk. However, these claims have been proven to be false as scientific reports disapproved these claims and no significant decrease in the nutritional value of pasteurized milk has been reported when compared with raw cow milk (Jose *et al.,* 2015); rather, a risk reduction and a safer hygienic milk as regards pasteurized milk consumption. Raw cow milk can contain harmful bacteria that may lead to serious illness. Most countries like Australia, Canada and Scotland have banned raw cow milk for human consumption. It is prohibited in 20 American states while other states restrict its sales. In Northern Nigeria and many other parts of Africa, raw cow milk and milk products are traditionally stable food commodities for the nomadic population (Okekechukwu *et al.,* 2020).

2.2 Milk Composition and Nutritive value

Milk provides essential nutrients and it is an important source of dietary energy, high quality proteins and fats. The principal constituents of milk are water, fats, proteins, lactose (milk sugar) and minerals (salts). Milk can make a significant contribution to the required nutrients intake for calcium, magnesium, phosphorus, selenium and riboflavin (Yusha'u, 2018). The species of dairy animal, its breed, age and diet, stage of lactation, number of parturitions, farming system and physical environment as well as season are contributing factors that usually influence the flavor and composition of milk and thus allow the production of a variety of milk product (Yusha'u 2018; FAO 2019).

2.2.1 Water

Milk contains a lot of moisture content (81.33%-83%), high moisture content is directly proportional to high water activity which relatively supports microbial growth (Londhe *et al*., 2012; Dandare *et al*., 2014).

2.2.2 Carbohydrate

Lactose is the main carbohydrate of milk. It is formed by the union of one molecule of D-galactose (engaged by its semiacetyl function) and one molecule of D-glucose (committed by its hydroxyl 4 position). It has a β-galactoside 1,4 bond (which is hydrolyzed by a β-galactosidase) and is a 4-Dglucopyranosyl-β-D-galactopyranose (Rutherfurd *et al*., 2015)

2.2.1 Proteins

Proteins in milk are of great quality, they contain all the essential amino acids, and elements that our bodies cannot produce. Proteins are the building blocks of all living tissue (Rutherfurd *et al*.,

2015). During the gastric digestion of the whole milk, the fat globules are physically entrapped within the protein clot formed, the nature of the protein network formed will influence the rate of release and also the digestion of fats by gastrointestinal lipases (Ye *et al*., 2019).

2.2.2 Fat

In milk, fat is the main source of energy. Fat is present in milk in the form of an emulsion of fat cells; the concentration of the fat in milk can be found in small cells called globules suspended in water which varies considerably with breed and composition of feed (Dandare *et al*., 2014; Roy *et al*., 2020). Approximately, about two thirds of the fats in milk is saturated, which plays a number of key roles in our bodies from construction of cell membranes and key hormones to providing energy storage and nutrient delivery during digestion (Roy *et al*., 2020).

2.2.3 Vitamins

Levels of vitamin A, D and E are variable in raw milk, depending on the season as there is a slight increase during the pasture season (dry-wet). They are fat-soluble, so it is found in fat and can be lost during skimming. Other vitamins are water soluble, in the case of ascorbic acid (C), it is present in small quantities in fresh milk and is destroyed by contact with air and also during pasteurization (Yusha'u 2018).

2.2.4 Minerals

They play an important role in the structural organization of casein micelles, they are; potassium, sodium, calcium, magnesium. Calcium, one of the essential minerals in raw cow milk helps in reduction of cancers, particularly colon cancer. It also helps lower risk of osteoporosis and fractures in older adults, formation of strong teeth and reduction of dental cavities and lowers the risk associated with kidney stones development (Adesina *et al.,* 2012; Yusha'u 2018).

2.2.5 Enzymes

Enzymes are specific globular proteins produced by living cells. Each enzyme has its isoelectric point and is susceptible to various denaturing agents such as pH change, temperature, ionic strength, organic solvent. The enzymes assist in gastrointestinal digestion of the coagulum formed by the fats to the casein-rich protein models (Ye *et al*., 2017; Mulet-Cabero *et al*., 2020).

2.3 Contaminants in Fresh raw cow milk

The quality of fresh raw cow milk can be affected by pathogen contamination, chemical additives, nutrient degradation and environmental pollution. Microbiological hazards are a major food safety concern in the dairy sector because fresh raw cow milk is an ideal medium for the growth of bacteria and other microorganisms. These contaminants can be introduced into the fresh raw cow milk from the environment, by the herd handlers or directly from the dairy animals themselves. Fresh raw cow milk can contain harmful microorganisms such as *Salmonella*, *Escherichia coli* 0157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Bacillus cereus*, *Clostridium botulinum*, *Mycobacterium bovis* and *Brucella abortus*. A variety of mesophilic, thermophilic and psychrophilic microorganisms are predominant in fresh raw cow milk. While mesophilic bacteria are temperature dependent and are lost during pasteurization, thermophilic bacteria may survive pasteurization (FAO 2019).

Chemical hazards can be unintentionally introduced into the fresh raw cow milk, making them unsafe and unsuitable for consumption. Milk can be contaminated when the milking animals are feeding, the water used on farms could also contain chemical residues, unclean environment and improperly sanitized milk storage facilities could also introduce contaminants directly or indirectly

into the fresh raw cow milk. Chemical hazards include detergents, teat disinfectants, dairy sanitizers, anti-parasitic, antibiotics, herbicides, pesticides and fungicides (FAO 2019).

Refrigeration of raw cow milk, while preventing the growth of non-psychrotrophic bacteria, will select for psychrotrophic microorganisms that are already present in the fresh raw cow milk. These psychrotrophs, gained access into the fresh raw cow milk from soiled cows teat, dirty equipment and the environment. Minimizing the level of contamination from these sources will help prevent psychrotrophs from growing to significant levels in fresh raw cow milk during the on-farm storage period or at the processing plant. The longer fresh raw cow milk is held before processing (legally up to 5 days), the greater the chance that psychrotrophs will increase in numbers greatly (Eliandra et al., 2018).

2.4 Sources of Microbial Contamination of Fresh raw cow milk

Generally, microbial contamination of fresh raw cow milk can be from the internal and or external sources.

2.4.1 Interior of Udder

Varying numbers of bacteria are found in aseptically drawn fresh raw cow milk with the reported count of <100-10,000 CFU/ml from normal udder, but an anticipated average is 500-1,000CFU/ml in advanced countries. Microorganisms enter the udder through the duct at the teat tip that varies in length and its surface is heavily keratinized (Eliandra et al., 2018). This keratin layer retains the milk residues and exhibit antimicrobial activity. Microorganisms dislodge particularly from teat canal during the milking process, different species of bacteria present in the udder of infected animal can also be dislodged into the fresh raw cow milk. Micrococci, Corynebacterium, Streptococci, Staphylococci, Bacillus and some enteric microorganisms have been reported to be found in fresh

raw cow milk which are also major cause of mastitis and sub-clinical mastitis (Cancino-Padilla *et al*., 2017). The influence of mastitis on the total bacteria count of fresh raw cow milk depends on the type of the infecting microorganism, the stage of the infection and the percentage of the herd infected.

2.4.2 Exterior of Udder

The microorganisms that are naturally associated with the skin of the animals as well as those derived from the environment where the cow is housed and milked are predominant in contaminated raw cow milk (Mbuk *et al*., 2016). Contaminations of fresh raw cow milk from soiled udder and teat with mud, dung, bedding materials such as sawdust, straw are possible when proper cleaning and sanitizing procedure are not practiced by herd handlers during pre-milking and post milking operations (Okechukwu *et al*., 2020). The bedding material during winter has high number of bacteria, mainly psychrotrophs, coliforms and *Bacillus* species, these bacteria can be introduced indirectly into the fresh raw cow milk. Generally, psychrotrophic and thermoduric bacteria usually predominate on the teat surfaces which could be introduced into raw cow milk, these bacteria have been reported to sometimes survive pasteurization when improperly done. The cow skin serves as a reservoir of contributing bacteria directly to fresh raw cow milk, the hairs around the udder, flanks, tail may indirectly contribute microbes into the air, especially *Bacillus species*, which could contaminate the fresh raw cow milk during or after the milking processes (Eliandra *et al*., 2018).

2.4.3 Milking Personnel

The individual hygiene status of the personnel handling the cows at different stages of fresh raw cow milk production plays a pivotal role in maintaining hygiene and preventing fresh raw cow milk contamination. Risk of contamination are higher when cows are hand milked compared to when

milked by machine. Hand contact with feeds, dust, bedding materials shortly prior to milking; the clothes, sweat and saliva of the personnel during the milking process all have a great influence on the rate of contamination of the fresh raw cow milk (Okechukwu *et al*., 2020).

2.4.4 Milking Equipment

Improperly cleaned milking and cooling equipment are one of the main sources of fresh raw cow milk contamination (Okpo *et al*., 2017; Gunasena and Siriwardhana, 2021). Milk residues left on the equipment surfaces supports the growth of a variety of microorganisms. It is therefore necessary to use equipment with smooth surfaces and minimal joints which should be washed thoroughly and sterilized appropriately. The tanker and collecting pipes are also potential sources of contamination, if not adequately cleaned and sanitized (Gunasena and Siriwardhana, 2021).

2.4.5 Water supplies

Water used in production should be ascertained to be of good bacteriological quality. Untreated water supplies from natural sources like bore holes,wells and rivers, are sometimes contaminated with faecal microorganisms (Coliforms, Streptococci and Clostridia), saprophytic bacteria (*Pseudomonas species*, Coliforms, *Bacillus* spores, C*oryneform* bacteria, other gram-negative rods and lactic acid bacteria), and may serve as potential source of contamination to raw cow milk and sometimes be a leading cause of mastitis in cows (Mbuk *et al*., 2016).

2.4.6 Airborne contamination

Aerosol dispersed into air while milking cows and personnel activities during the milking process is insignificant in comparison to microbes that are derived from teat surfaces. However, continuous dispersal of dust, moisture and bacteria in air and its incorporation in raw cow milk leads to greater level of contamination (Eliandra *et al*., 2018). Activities like sweeping of floors, brushing of

animals, handling hay and feeds, accumulation of dust and dirt on walls and ceiling shed prior to milking are all but some practices that contribute greatly to increase in total aerobic and coliform count in raw cow milk (Suranindyah *et al*.,2015; Eliandra *et al*., 2018).

2.5 Microbial Analysis on Fresh raw cow milk

Quality assessment on fresh raw cow milk is mandated by the authorized organizations in ensuring production of "Grade A" milk. Federal Grade A Pasteurized Milk Ordinance safety standards are procedures documented to guide through on farm problems/deficiencies associated with abnormal high aerobic count and poor quality raw cow milk production (PMO, 2009; FAO 2019). The primary methods used to assess fresh raw cow milk quality include: Somatic Cell Count, Standard Plate Count, Preliminary Incubation Count and Methylene Blue Reductase test.

The microbial content of milk indicates the hygienic levels during milking process, activities which include cleanliness of the milking utensils, proper storage and transport as well as the udder preparations of the individual cow.

2.5.1 Standard Plate Count

Standard Plate Count (SPC) is one of the most commonly used microbial quality tests for milk and milk products, it involves total aerobic bacteria count in the milk product (FAO 2019). Aseptically collected milk from clean, healthy cows generally has SPC values of less than 1,000. Higher counts suggest that contaminating bacteria are entering the milk from a variety of possible sources. Although it is impossible to eliminate all sources of contamination, counts of 10,000 or less should be achievable by most farms. One of the most frequent causes of high SPC is poor cleaning of the milking system. Milk residues on equipment surfaces provide nutrients for growth and multiplication of bacteria that can then contaminate the milk of subsequent milking (Eliandra *et al*.,

2018). Coliforms are often used as indicators of faecal contamination; they are strains that commonly exist in the environment. Coliforms may enter the milk supply as a consequence of milking soiled cows or dropping the milking claw into manure during milking. Generally, counts >50 would indicate poor milking hygiene or other sources of contamination. Higher coliform counts more often result from dirty equipment and in rare cases result from milking cows with environmental coliform mastitis.

2.5.2 Methylene Blue Reductase Test

Methylene Blue Reductase test (MBRT), is used as a rapid method to assess the microbiological quality of raw and pasteurized milk. This test is based on the fact that the dye solution added to the milk gets decolorized when the oxygen present in the milk gets exhausted due to microbial activity. The sooner the milk decolorizes, the greater the microbial activity and thus the milk is assumed to be of a low bacteriological quality (Dharani *et al*., 2021). MBRT is widely used as a rapid microbiological assay in major dairy farms around the world and also serves as a criterion for the raw and processed milk acceptance or rejection (Table 2.1).

Table 2.1: Fresh raw cow milk Quality

Time	**Grade**
5 hours and above	Very good
3 to 4 hours	Good
1 to 2 hours	Fair
Less than 30 min	Poor

Source: Food and Agriculture Organization of United Nations 2019

2.5.3 Somatic Cell Count

The Somatic Cell Count (SCC) is one of the main indicator of fresh raw cow milk quality. The majority of the Somatic cells are leukocytes (white blood cells) which becomes present in increasing numbers in the fresh raw cow milk as an immune response to a mastitis causing pathogen in cows and also epithelial cells which are shed within the udder of an infected cow. The SCC is usually quantified as the number of cells per ml of fresh raw cow milk. An individual cow with SCC of 100,000 or less indicates an uninfected cow possibly sub clinical mastitis; a threshold SCC of 200,000 or above is at greater risk of being infected in at least one quarter of the udder; SCC of 300,000 or greater are cows infected with significant pathogens (FAO., 2019).

2.5.4 Preliminary Incubation Count

The Preliminary Incubation (PI) Count is a unique test that has the ability to detect bacteria that grow in cold environments (Psychrotrophic bacteria). The standard plate count of the fresh raw cow milk is done as at collection, the fresh raw cow milk is then refrigerated and held for 18 hours; and then, the standard plate count (SPC) is performed again. The PI count is then estimated by comparing the SPC done when the raw cow milk is still fresh with the SPC after refrigeration (FAO., 2019). If the PI count is higher than the initial SPC, it suggests some undesirable practice on the farm which allowed these bacteria gain entry into the fresh raw cow milk. These Psychrotrophs, could gain entry into the fresh raw cow milk from various sources; improperly cleaned equipment during milking process, hygiene of the cow and generally poor sanitary conditions of the environment (Eliandra *et al*., 2018).

2.6 Staphylococci

Staphylococci species are gram-positive, aerobic microorganisms appearing as spherical and form a grape-like clusters. Fresh raw cow milk provides an excellent medium for growth of *Staphylococci* due to its high nutritional content. This foodborne pathogen is considered as one of the world's leading causes of disease outbreaks related to food consumption and it is also responsible for a variety of manifestations and diseases (Jamali *et al.,* 2014; Aliyu *et al*., 2019).

Staphylococcal food poisoning is caused by consumption of staphylococcal enterotoxins (SE) formed by *Staphylococcus aureus* in food. More than 20 different SE and SE-like super antigens have been described (Hennekinne *et al.,* 2012), but only a few have been demonstrated to elicit an emetic response in a monkey feeding assay. Staphylococcal enterotoxins that have been shown to exhibit emetic activity include the classical Enterotoxins SEA, SEB, SEC, SED, and SEE, and to a limited degree (Thomas *et al.,* 2007).

Mastitis is considered the most prevalent disease in dairy cow and is endemic in all dairies (USDA-APHIS, 2016). Coagulase negative *Staphylococcus* (CNS) species, variously referred to as non-aureus *Staphylococcus* (NAS), are currently the most prevalent intra-mammary pathogen of lactating dairy cow, and as many as 10 different species have been identified in this group (Tenhagen *et al.,* 2006; Thorberg *et al.,* 2009; Condas *et al.,* 2017). The most common isolates are *S. chromogenes, S. epidermidis, S. simulans, S. hyicus, S. xylosus, S. warneri, and S. equorum*. Intra-mammary infection (IMI) with CNS is generally associated with subclinical mastitis that may result in increased somatic cell count and occasional clinical mastitis or persistent infection that leads to reduced milk production (Pyörälä & Taponen, 2009; Tomazi *et al.,* 2015).

2.7 Enterobacteriacea

The Enterobacteriaceae are large, heterogeneous group of gram-negative rods whose natural habitat is the intestinal tract of humans and animals. The family includes many genera; *Escherichia, shigella, Salmonella, Enterobacter, Klebsiella, Serratia, Proteus* and others. Some of these organisms such as *Escherichia coli,* are parts of the normal microbiota and incidentally cause disease, but others, the *Salmonella species* and *Shigella species* are regularly pathogenic for humans (Mbuk *et al*., 2016).

Coliforms are defined as aerobic or facultatively anaerobic, gram-negative, non-spore-forming rods capable of fermenting lactose, resulting in gas and acid production within 48 h at 35°C (Nornberg *et al.,* 2010). Detection of coliforms plays an important role in the dairy industry because coliforms are frequently used as hygiene indicators and there are clear regulatory limits for the presence of coliforms in finished dairy products. For example, the US Pasteurized Milk Ordinance limits the number of coliforms in pasteurized grade "A" milk to ≤10 cfu/ mL (FDA, 2011). Coliform bacteria that are psychrotolerant and capable of growing at refrigerated storage temperatures are of particular concern for the dairy industry, as psychrotolerant growth can result in physical degradation and unacceptable sensory characteristics of the product due to the production of lipolytic and proteolytic enzymes (Nornberg *et al.,* 2010).

Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae and *Serratia marcescens* are four common coliform bacteria that cause mastitis (Junaidu *et al.,* 2011; Okpo *et al*., 2016). Coliform bacteria are normal inhabitants of soil, digestive tract and manure. They multiply and accumulate in contaminated bedding. Coliform numbers of 1,000,000 or more per gram of bedding increase the likelihood of an udder infection and clinical mastitis (Podder *et al.,* 2014). *Klebsiella pneumoniae* is common in sawdust bedding, especially rough-cut sawdust that contains bark or soil (Omabarak

and Elbagory, 2017). Coliforms invade the udder through the teat sphincter when teat ends come in contact with coliform bacteria. Once coliform bacteria enter the mammary gland, they either multiply rapidly or remain dormant and pose future treat to the health of the cow, however, the immune response of the cow is highly successful in destroying these bacteria. The bedding used to house cow is the primary source of environmental pathogens, but contaminated teat dips, intramammary infusions, water used for udder preparation before milking, water ponds or mud holes, skin lesions, teat trauma, and flies have all been incriminated as sources of infection (Kivaria and Noordhuizen, 2007). A study carried out in Sokoto State, Nigeria reported *E. coli* (9.78%), *Klebsiella species* (4.35%), *Proteus species* (8.69%) and *Enterobacter species* (1.09%) as the most commonly found pathogens in raw milk (Junaidu *et al.,* 2011). Similarly, in a study conducted using raw cow milk consumed in Khartoum, Sudan, coliform isolated include; *E. coli (*32%), *Enterobacter species* (29.2%), *Klebsiella species* (19.4%), *Serratia species* (11.1%) and Citrobacter (1.0%) (Salman and Hamad, 2011).

2.8 Helicobacter specie

Helicobacter pylori is a Gram-negative, microaerophilic bacterium. It has been detected in half of the human population (Ghoshal *et al.,* 2010; Garza-González *et al.,* 2014; Hooi *et al.,* 2017). Prevalence of *H. pylori* infection varies widely according to geographic area, age, race, and ethnicity (Gharavi, *et al.,* 2016). *H. pylori* has a strong affinity to stomach mucosa and induce serious diseases in the gastrointestinal tract such as chronic gastritis, duodenal ulcer, and gastric cancer (Graham, 2014). Hence, the World Health Organization considers *H. pylori* as an important Class I carcinogen factor (Testerman and Morris, 2014). Even though humans are the principal reservoir of *H. pylori* (Brown, 2000; Payao and Rasmussen, 2016), it could spread through food and water by fecal-oral

and oral-oral routes and colonize the stomach and intestines of humans and several animal species (Adler, 2014; Payao and Rasmussen, 2016).

Professionals dealing with animals and animal products food such as veterinarians, butchers and slaughterhouse staff showed high levels of antibodies against *H. pylori* (Fox and Wang, 2014; Talaei *et al.,* 2015), suggesting that animal might be a source of contamination for humans. *H. pylori* has been isolated from milk of different farming animals mainly cow, ewe, camel, and sow (Quaglia, *et al.,* 2008; Talaei *et al.,* 2015). These findings confirm that farming animals are a potential source of *H. pylori* and represent a risk of contamination for humans handling them or consuming their originated products such as meat and milk. Therefore, cow milk which remains the most consumed milk in the world, it is most likely that it may represent a potential source of human infection by *H. pylori.*

Helicobacter pylori (H. pylori) infection is widespread in humans; it is present in 20 to 50% of the population in developed countries and 80% of the population in developing countries (Atherton and Blaser 2009). Most infected individuals display only asymptomatic gastritis, whereas a small proportion develop severe disease, including peptic ulceration and gastric malignancy. *Helicobacter pylori* have been considered as a main cause of mucosa associated lymphoma, peptic ulcer disease, type B gastritis, and gastric adenocarcinoma. The severity of clinical complications caused by *H. pylori* depends on the presence of virulence genes which is the most important factor responsible for *H. pylori* infections. Vacuolating cytotoxin (*vacA*) is one of the most important virulence factors in the occurrence of human clinical diseases caused by this bacterium. The *vacA* belongs to the group of genes with variable genotypes or structures. The *vacA* gene is present in virtually all strains of *H. pylori* but it is polymorphic, comprising variable signal regions (type *s1* or *s2*) and mid-regions (type *m1* or *m2*). The *s*1 type is additionally subtyped into *s1a*, *s1b* and *s1c* subtypes and the *m1* into

m1a and *m1b* subtypes (Pinto-Ribeiro *et al.,* 2016; Román-Román *et al.,* 2017). The mosaic combination of s- and m-region allelic types determines the particular cytotoxin and, consequently, the pathogenicity of the bacterium. Another well-characterized virulence factor is the cytotoxin-associated antigen (*cagA*), which is encoded by one of the genes located in the *cag* pathogenicity island (PAI) (Censini *et al.,* 1996; Feliciano *et al.,* 2015). Strains expressing *vacAs1* and/or *cagA* are present at a higher frequency in patients with duodenal ulcers, atrophic gastritis, and gastric carcinoma (Eck *et al.,* 1997; Feliciano *et al.,* 2015) and are referred to as type I strains. In contrast, type II strains, which lack the *cagA* gene, present a nontoxic form of *vacA* and are considered less virulent (Xiang *et al.,* 1995; Feliciano *et al.,* 2015).

2.9 Foodborne Infections associated with Milk and Milk product

The leading Foodborne illness commonly associated with consumption of contaminated fresh raw cow milk and milk products include the following.

2.9.1 Listeriosis

Listeriosis is a bacterial infection most commonly caused by *Listeria monocytogenes*. The main route of acquisition of Listeria is through the ingestion of contaminated food product. It has been isolated from dairy product, meat vegetable, fruit and seafood (FAO, 2019). The severity of the illness could lead to severe sepsis, meningitis or encephalitis sometimes resulting to a lifelong harm and even death. Those at risk of severe illness are the elderly, unborn babies, new born and those who are immunocompromised (Valente *et al*., 2019). In pregnant women, it may cause stillbirth or spontaneous abortion, or preterm birth of the baby. Generally, Listeriosis causes mild, self -limiting gastroenteritis and fever in people; an inflammation of gastrointestinal tract of the stomach and small intestine leading to severe diarrhea, vomiting and abdominal pain (Valente *et al*., 2019).

2.9.2 Salmonellosis

This is an infection by *Salmonella species* commonly caused by consumption of contaminated food or water (Valente *et al*., 2019). Both young and old are at risk of being infected but the severe cases observed in children, elderly, people with AIDS, people with compromised immune systems are fatal if prompt medical treatment is not received. Salmonellosis is usually accompanied with diarrhoea, fever, chills, nausea, vomiting and abdominal cramping (CDC 2016).

2.9.3 Campylobacterosis

Campylobacter is a major cause of gastroenteritis throughout the world. The infection occurs mainly following consumption of contaminated undercooked poultry, contaminated dairy product or water. The most common symptoms of Campylobacter infection include diarrhea, abdominal pain, fever, nausea and vomiting. Treatment is usually replacement of electrolyte and fluid loss but antimicrobial may be needed to treat invasive cases and the carrier state of the bacterium.

2.9.4 *Escherichia coli* Infections

Escherichia coli is a bacterium present in the gut of humans and other warm-blooded animals. They are mostly harmless but some strain may become pathogenic and lead to severe food borne diseases (Valente *et al*., 2019). This bacterium is ubiquitous and is usually transmitted through consumption of contaminated water, food such as undercooked meat product and raw cow milk. The symptoms include abdominal cramps and diarrhoea which may be bloody, fever and vomiting. Most patient recover within 10 days, although in a few cases, the disease may become life threatening (CDC 2016).

2.9.5 Diarrhoeal Infection

Diarrheal disease is the third leading cause of infant and child mortality in developing countries (WHO, 2014; CDC 2016). In Nigeria, mortality among children below the age of 5 years from diarrhoea was 331.3 per 100,000 children in 2016, although there has been a reduction in mortality in this age group of over 20% between 2005 and 2015 (GBD 2017 & 2018). Among the etiologies associated with mortality due to diarrhoea in Nigeria in children under 5 years, the mortality data assessment per pathogen conducted by the research group estimated that rotavirus has the highest impact (45%), with cryptosporidiosis considered responsible for (14.3%). Other diarrhoea agents associated with death in this age group include *Adenovirus* (10.3%), *Shigella* (6.1%), *Salmonella species* (4.2%), *Norovirus* (3.6%), *Entamoeba histolytica* (2.6%), *Vibrio cholerae* (3.5%), enteropathogenic *E. coli* (3.3%) and *Campylobacter specie*s (2.1%) (GBD, 2017).

In most cases, diarrhoea can be managed at home and it will resolve itself in a few days. Prompt replacement of lost fluid to ensure infants and patients stay hydrated and following bananas, rice, applesauce and toast diet (BRAT) are to be observed to ease symptoms. The guidelines of WHO in treatment of diarrhoea explicitly discourages the use of antibiotics for treating acute diarrhoea as they will be of no effect in majority of the cases, due to non-bacterial etiology (WHO 2018). However, the use of antimicrobial agents should be restricted to cases of bloody diarrhoea and cholera cases with severe dehydration. Treatment guidelines include:

1) Low-cost supportive interventions; Oral rehydration solution (ORS), intravenous fluids, continuous feeding/breastfeeding, use of zinc tablets.
2) Community based management practices by parent/ guardians; ensuring patient are well hydrated and proper hygiene practiced.

Efunshile *et al.* (2019) reported using supportive treatment (ORS, intravenous fluid (IVF), zinc tablets and vitamin A) for children below age of 5 years presented with watery diarrhoea at Federal Medical Center, Abakaliki, Nigeria. About 86.9% of the children received antibiotics and over 30% of the children received probiotics in the health care setting. Ciprofloxacin was used mostly for 72.4% of the children, metronidazole for about 30.2% and gentamycin for about 15.1%. In some cases, a combined therapy was used with ciprofloxacin and metronidazole in 22% of the children.

2.9.6 Gastrointestinal Infection

These are bacterial or viral infections, symptoms most commonly are diarrhoea (may be bloody), nausea, vomiting, fever and abdominal pain (WHO 2018). The treatment options include staying hydrated and taking antibiotics due to severe cases of diarrhoea. Antimicrobials used in treatment include penincilin, cephalosporin, antifolate or sulfa combinations, nitro imidazole, glycopeptide and monobactam (WHO 2018).

2.9.7 *Staphylococcal* food poisoning (SFP)

Staphylococcal food poisoning (SFP) is one of the most prevalent causes of foodborne intoxication worldwide. Scallan *et al.* (2011), reported an estimated cases of 241,148 and 6 death in the United States alone in 2006. SFP is caused by consumption of staphylococcal enterotoxins (SE) formed by *Staphylococcus aureus* in food product. More than 20 different SE and SE-like super antigens have been identified (Hennekinne *et al.,*2012). High prevalence of *Stapylococcus aureus* in humans and animals has been reported and thus this bacterium persistently colonizes the anterior nares of 20 to 30% human population and livestock, it has also been isolated from a wide range of food products (Baumgartner *et al.,* 2014). Johler *et al.* (2015), reported SFP outbreak in 10 children that consumed cheese made from raw cow milk in Swiss boarding school with an average incubation time of 4 and

half hour which is also depended on the age of the patient. Within 2 and half hour after consumption, 2 of the youngest children complained of abdominal pain, fever and aching limbs that progressed quickly to emesis, followed by severe diarrhoea.

2.10 Antimicrobials Therapy

Food borne related infections mostly present symptoms from vomiting, cramping, gastroenteritis to mild or severe diarrhoea. Majorly, children, pregnant women, elderly and immunocompromised individuals are at greater risk of food poisoning infections. Acute infectious diarrhoea remains a very common health problem, even in the industrialized world. One of the dilemmas in assessing patients with acute diarrhoea is deciding when to test for etiological agents and when to initiate antimicrobial therapy (Fig. 2.1). As at onset of infection, antimicrobial treatment is not required in most patients with acute gastroenteritis or diarrhoea because, usually, the illness is self-limiting as the body mechanisms can regulate and flush out the toxins. Prompt rehydration with electrolytes solutions and/or use of intravenous fluids is usually all that is required to control the infections. Unnecessary antimicrobial therapy can also lead to adverse events, and could add to antimicrobial resistance development (Okechukwu *et al*., 2020).

However, antimicrobial treatment tends to quicken the clinical resolution of diarrhoea in some instances whereby severe, prolonged and potentially complicated cases arise. It also prevents the progression of disease and reduce the severity of associated symptoms, such as fever, abdominal pain and vomiting. Furthermore, antimicrobial therapy decreases secondary cases, by halting person-to-person spread of most pathogens; in health care settings, special consideration for the use of antibiotics in the treatment of child-care workers, health professionals and workers in the catering industry or services with severe diarrhoea within 2-3 days is necessary to avoid cross infections. In

such cases, prompt adoption of empirical antimicrobial therapy is useful and recommended by the World Health Organization (Giannattasion *et al.,* 2016).

Antimicrobial mostly used in the empirical therapy in treating related food borne infections in adult and sometimes children include: Penicillin, cepalosphorin, fluroquinolones, trimethoprim-sulfamethoxazole, nitroimidazole, macrolide, tetracycline, glycopeptide and monobactam.

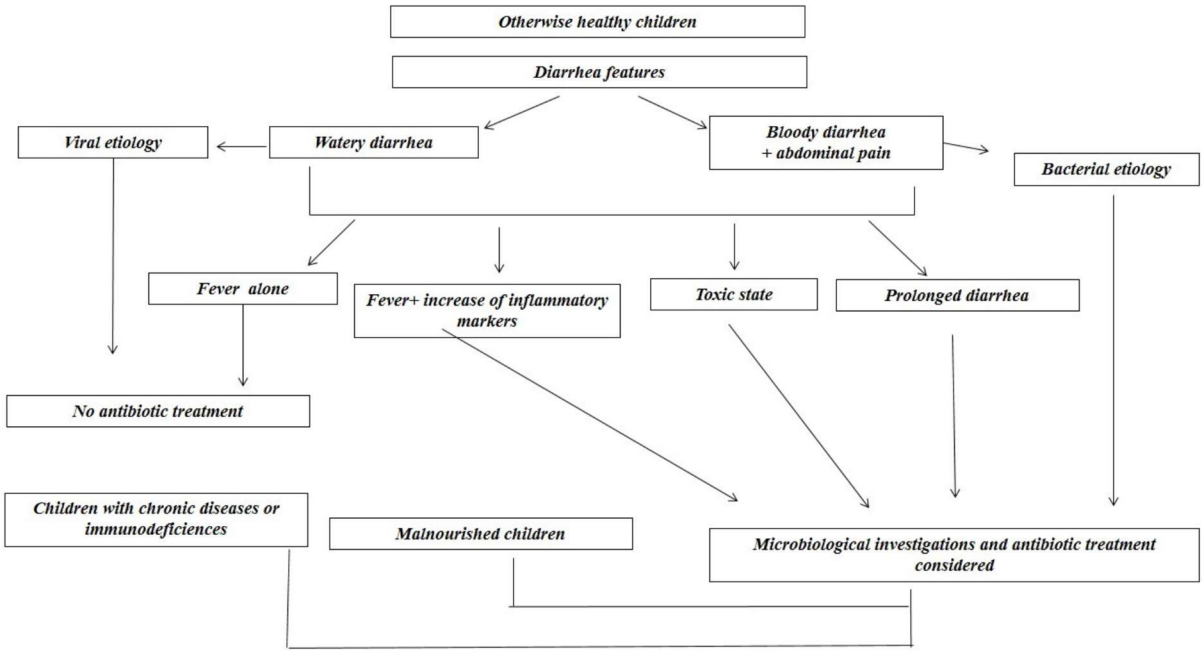

Figure 2.1: Criteria to decide antibiotic treatment in children with infectious diarrhoea.
Source: World Health Organization (WHO)

2.10.1 B- Lactams

Beta-lactam antibiotics are bactericidal and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in Gram-positive organisms, being the outermost and primary component of the wall.

The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by D-alanyl-D-alanine transpeptidases which are penicillin-binding proteins (PBP).

Amoxicillin, particularly, has broad spectrum activity against both Gram positive and Gram-negative bacteria. *Streptococcus specie, Bacillus subtilis, Enterococcus specie, Haemophilus specie, Helicobacter specie,* and *Moraxella specie* show variable sensitivity to amoxicillin.

The cephalosporins are grouped into to five generations based on their introduction, and spectrum of activity on Gram negative and Gram-positive bacteria. The third generation cephalosporins have equally wide antimicrobial activity spectrum and fewer adverse effects than the fluoroquinolones, they have been considered by many as the best drugs for the empirical treatment of severe acute infectious diarrhoea in children; particularly, ceftriaxone and in some cases, ceftazidime are used intravenously or intramuscularly for the treatment of severe bacterial infections (Etebu and Arikekpar 2016)

2.10.2 Macrolides

Erythromycin, a macrolide, inhibits growth of bacteria by binding to the 50s subunit of the bacterial 70s rRNA complex. Protein synthesis and subsequent structure and functional processes critical for life or replication are inhibited. Erythromycin interferes with aminoacyl translocation, preventing the transfer of the tRNA bound at the A site of the rRNA complex to the P site of the rRNA complex (Arenz and Wilson, 2016). This interferes with the production of functionally useful proteins, which is the basis of this antimicrobial action. Erythromycin is also a good option for the treatment of severe cases of cholera in young children who should not take tetracyclines or fluoroquinolones (Etebu and Arikekpar 2016).

2.10.3 Tetracycline

Tetracyclines are a group of broad-spectrum antibiotic compounds that have a common basic structure and are either isolated directly from several species of Streptomyces bacteria or produced semi-synthetically from those isolated compounds. Its molecules comprise a linear fused tetracyclic nucleus (ring designated A, B, C and D) to which a variety of functional groups are attached. Tetracyclines are named for their four (tetra) hydrocarnon rings (-cycl-) derivation (_ine). They are growth inhibitors (bacteriostatic) and are only effective against multiplying microorganisms.

Their target of antimicrobial activity in bacteria is the ribosome. They disrupt the addition of amino acids to polypeptide chains during protein synthesis. In spite of their low cost and broad antimicrobial spectrum, the use of tetracyclines in pediatric patients is limited by permanent dental discoloration in children younger than eight years of age. Additionally, tetracyclines have been shown to cause enamel hypoplasia and reversibly impair bone growth. Because of these important side effects, tetracyclines have been progressively displaced by safer, equally effective drugs, for the treatment of most conditions in which they are likely to be effective (Etebu and Arikekpar 2016). The preferred tetracycline is doxycycline, because the risk of dental staining is less with this drug than with the other tetracyclines; in addition, it is given only twice a day. In the treatment of tetracycline-resistant strains, TMP-SMX has been used for children less than eight years of age who have cholera; ampicillin and macrolides may be reasonable alternatives (Etebu and Arikekpar 2016).

2.10.4 Fluoroquinolones

The quinolones, also known as fluroquinolones are synthetic bactericidal drug with broad spectrum activity against both gram-positive and gram-negative microorganisms. The quinolones function

by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV enzymes necessary to separate bacterial DNA strands, thereby inhibiting cell division (Guan *et al.,* 2013).

Ciprofloxacin, which is a second-generation fluoroquinolone is the most commonly used, while levofloxacin (a newer generation) has broader activity against Gram positive organisms (Aldred *et al.,* 2014). Its spectrum of activity includes most strains of bacterial pathogens responsible for respiratory, urinary tract, gastrointestinal, and abdominal infections (Kim and Hooper, 2014).

The fluoroquinolones are mostly the drugs of choice for the empirical treatment of acute diarrhoea in adults, because they are active against most of the common entero-pathogens and have excellent tissue and intracellular penetration, they are also suitable for oral administration, and have a favorable safety profile in adults. However, the use of fluoroquinolones in a child and adolescent is restricted to special circumstances after careful assessment of the risks and benefit to the individual has been considered, and/or in consideration of the severity of infections caused by multidrug-resistant pathogen for which no safe or effective alternative is known, and/or used as second line therapy where first line therapy has failed (Murray and Baltimore, 2007; Etebu and Arikekpar 2016).

2.10.5 Aminoglycosides

They inhibit bacterial synthesis by binding to the 30S ribosome and act rapidly as bactericidal antibiotics, they are usually given intravenously. The aminoglycoside antibiotics includes gentamicin, tobramycin, amikacin, kanamycin, and streptomycin. Gentamicin is a bactericidal antibiotic that works by irreversibly binding to the 30S subunit of the bacterial ribosome, interrupting protein synthesis ((Etebu and Arikekpar 2016). Gentamicin is broad spectrum and most effective against aerobic gram-negative rods, it can also be used in combination with other

antimicrobials to treat infections caused by gram-positive microorganisms such as *Staphylococci species* and some strain of *Streptococci species*.

2.10.6 Phenicols

The rising resistance rates, uncomfortable posology, and the risk of side effects, have contributed to the displacement of chloramphenicol as a good drug for the empirical treatment of acute diarrhoea. Nevertheless, it still may be used empirically if typhoid fever is strongly suspected on clinical grounds, as long as it is supported by up-to-date knowledge of antimicrobial susceptibility pattern of locally circulating strains. The use of chloramphenicol for the treatment of typhoid fever is associated with reduced mortality and decreased incidence of life-threatening complications, but the need for a long two – three-week regimen to prevent relapse and prolonged fecal shedding of pathogens is a significant drawback.

2.10.7 Metronidazole

In children with chronic conditions, metronidazole provides an alternative antimicrobial treatment option against gastroenteritis and diarrhoea. Oral metronidazole can be considered for sequential therapy after parenteral administration (Giannattasion *et al.,* 2016). However, all the international guidelines recommend microbiological examination and to start metronidazole or ciprofloxacin in Inflammatory Bowel Disease children with diarrhea recurrence.

2.10.8 Antimicrobial regime on *Helicobacter pylori*

The most widely recommended anti–*H.pylori* regimen has been a combination of a proton pump inhibitor and two of amoxicillin, clarithromycin, and metronidazole or tinidazole (known as “legacy triple therapy”). Initially, this was a highly successful regimen, but worldwide resistance to clarithromycin has been increasing, such that treatment success is now generally 80% or less

(Graham and Fischbach 2010). The best advice for clinicians is to prescribe only what is known to work locally and to routinely perform noninvasive post-treatment testing to confirm cure and to determine if resistance has begun to undermine the current locally effective regimens. Factors that will assist the clinician in the choice of regimen include the patient's history of antibiotic use, allergies, cost and availability of the drugs.

Resistance to clarithromycin and fluoroquinolones (which may also be used in anti–*H.pylori* therapies) develops rapidly which may not be countered by increasing the dose or duration of therapy. In contrast, resistance to metronidazole or tinidazole can be partially countered by increasing the dose and duration of therapy and by adding a proton pump inhibitor (Graham and Fischbach 2010). Resistance to amoxicillin and tetracycline is uncommon and prior use of these drugs does not affect reuse and development of resistance.

2.11 Currently recommended regimens

Current successful empiric anti–*H. pylori* regimens consist of multidrug combinations typically containing four drugs. They can be divided into non-bismuth-containing and bismuth-containing therapies. The effective non-bismuth-containing quadruple regimens use amoxicillin, clarithromycin, metronidazole or tinidazole, and a proton pump inhibitor, in a variety of combinations. They are;

Concomitant therapy*:* Combination of four drugs, specifically amoxicillin 1 g, clarithromycin 500 mg, imidazole or metronidazole 500 mg and a proton pump inhibitor, all given twice daily for 10–14 days. A nitroimidazole twice daily may be added to a legacy triple therapy dose pack if available.

Sequential therapy*:* Amoxicillin 1 g and a proton pump inhibitor twice daily for five days, then clarithromycin 500 mg, tinidazole or metronidazole 500 mg and a proton pump inhibitor, all twice daily for five days (total 10 days of therapy).

Hybrid (sequential and concomitant) therapy*:* Amoxicillin 1 g and a proton pump inhibitor twice daily for seven days, then amoxicillin 1 g, a proton pump inhibitor, clarithromycin 500 mg and tinidazole or metronidazole 500 mg, all twice daily for seven days (total 14 days of therapy).

Bismuth quadruple therapy: Bismuth subsalicylate or subcitrate two tabs four times daily, tetracycline 500 mg four times daily (with meals and at bedtime), metronidazole or tinidazole 500 mg three times daily (with meals) and a proton pump inhibitor twice daily for 10 or, preferably, 14 days.

2.12 Antimicrobials Resistance

Antimicrobial resistance is the expected result of the interactions of many microorganisms with their environment. Most antimicrobial compounds are naturally produced molecules from certain microorganisms, and, as such, co-resident microorganisms have evolved ancient mechanisms to overcome their actions and ability to survive in a competitive environment. These organisms are often considered to be “intrinsically” resistant to one or more antimicrobials. Acquired resistance happens when microorganisms are no longer susceptible to previously exposed antimicrobials concentrations in which they were originally susceptible. As a result, the drugs become ineffective and infections persist in the body thereby increasing the risk of spread to others. New resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases and resulting in prolonged illness, disability or death depending on the severity. The development of acquired resistance can be the result of mutations in chromosomal genes or due to

the acquisition of external genetic determinants of resistance, which may likely be acquired from intrinsically resistant organisms present in the environment. These acquisition of foreign DNA coding for resistance determinants are mainly through horizontal gene transfer (HGT).

Mutational resistance arises through a subset of a susceptible bacterial populations to certain antimicrobials. These subset bacterial cells develop mutations in their genes that affect the activity of the antimicrobial, they are preserved and survived in the presence of the antimicrobial molecule. Once a resistant mutant emerges, the antimicrobial eliminates the susceptible bacterial populations and the resistant mutant predominate. Mutations resulting in antimicrobial resistance and/or altering the antimicrobial actions via one of the following mechanisms:

- Modification of the antimicrobial target (decreasing the affinity for the drug),
- Decrease in drug uptake;
- Activation of efflux mechanisms to extrude the harmful molecule;
- Global changes in important metabolic pathways via modulation of regulatory networks.

Acquisition of foreign DNA material through Horizontal gene transfer is mainly the important drivers of bacterial evolution and frequently responsible for the development of antimicrobial resistance. Microorganisms sharing the same ecological niche with antimicrobial producing organisms harbor intrinsic genetic resistance determinant, these environmental resistome serve as a prolific source for the acquisition of antimicrobial resistance genes in clinically relevant bacteria and also, it has been implicated in the dissemination of resistance to many frequently used antimicrobials. Classically, bacteria acquire external genetic materials through three main strategies:

- Transformation (incorporation of naked DNA)
- Transduction (phage mediated)

- Conjugation

Transformation is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material from its surrounding through the cell membrane. Transduction involves introducing a foreign DNA into a bacterial or mammalian cells by a virus or viral vector. This process does not require physical contact between the cell donating DNA and the cell receiving the DNA. Mostly bacteriophages genome (viruses that infect bacteria) are used to package the resistance bacteria gene which is then introduced into another bacterium. Conjugation is a very efficient method of gene transfer that involves cell-to-cell contact and is likely to occur at high rates in the gastrointestinal tract of humans under antimicrobial treatment. It uses mobile genetic elements (MGEs) as vehicles to share valuable genetic information and direct transfer from chromosome to chromosome has also been well characterized (Manson *et al.,* 2010). The most important MGEs are plasmids and transposons, both of which play a crucial role in the development and dissemination of antimicrobial resistance among clinically relevant microorganisms.

CHAPTER THREE

3.1 MATERIALS AND METHODS

3.1.1 Materials

3.1.1.1 Equipment

- Incubator (Natural appliance: Aheinicke Company Portland, Oregon, U.S.A.
- Hot air oven (Baird and Tatlock London limited),
- Refrigerator (NAPCO Model 630 Portland, Oregon, U.S.A.)
- Autoclave (Adelphi MFG Co Ltd, Portland autoclave),
- Colony counter (NAPCO Model 630 Portland, Oregon, U.S.A.),
- Microscope (Wild M11, Switzerland),

3.1.2 Culture Media

Bacteriological media such as Peptone Water, Nutrient Agar, MacConkey Agar, Mannitol salt Agar, Blood Agar, Urea Agar, Brain Heart Infusion Agar and Broth

3.1.3 Reagents

Chemical reagents such as Lugol's iodine (May and Baker Ltd. Dagenham England), Crystal Violet, Carbol Fushin (May and Baker Ltd. Dagenham England), Acetone, oil immersion (BDH), Methylene blue, Hydrogen peroxide, Oxidase reagent, and Urea reagent.

3.1.4 Glass wares

Universal bottles, Microscope glass slides and Petri dishes (Pyrex, England), Pipette, Glass rod, Test tubes (Pyrex, England), Measuring Cylinder (Pyrex, England), Beakers (Pyrex, England), conical flask (Pyrex, England).

3.1.5 Antimicrobial Discs

Selected antibiotics used in this study are those commonly prescribed for food borne infections in Zaria metropolis. These include:

Amoxicillin- clavulanic acid AMC (30 µg), Ampicillin AMP (10 µg), Ceftazidime CAZ (10 µg), Cefoxitin FOX (30 µg), Ciprofloxacin CIP (5 µg), Gentamicin CN (10µg), Tetracycline TET (30 µg), Chloramphenicol CHL (30 µg), Erythromycin ERY (15µg) and Metronidazole MTZ (10 µg).

3.2 METHODS

3.2.1 Study Area

Fresh raw cow milk samples for this study were taken from four farm locations in Zaria.

a) Zango, Sabon-Gari L.G.A;

b) Jamaa Lima, Sabon-Gari L.G.A;

c) Kufena, Zaria L.G.A and

d) NAPRI, Shika-Giwa L.G.A

Questionnaire were used to evaluate the level of education of the handlers and also record the daily practices held by dairy farmers.

Inclusion Criteria: Healthy cows that are lactating on the farm and Handlers that gave consent for analysis.

Exclusion Criteria: Non lactating cows, unhealthy cows and Handlers that did not give consent for analysis.

Table 3.1 below shows the set of primers used in this study, the sequences, sources, amplicon sizes as well as the polymerase chain reaction time and conditions.

Table 3.1: Set of Primers

Primer	Sequence	Product size(bp)	PCR conditions	Source
mecA	F: 5' AAA ATC GAT GGT AAA GGT TGGC 3' R: 5'AGT TCT GCA GTA CCG GAT TTGC 3'	533	94ºC/ 1min;58ºC/ 1min; 72ºC/ 2 min: 72ºC/ 5 min	Pereira *et al.,* (2009)
ermB	F: 5' GAAAAGGTACTCAACCAAATA 3' R: 5' AGTAACGGTACTTAAATTGTTTAC 3'	639	94°C/5 min× 30; 94°C/60s; 41°C/60s;72°C 40 s);7 min.	Zhang *et al.,* (2016)
nim	F: 5'ATGTTCAGAGAAATGCGGCGTAAG CG 3' R: 5' GCT TCC TTG CCT GTC ATG TGC TC 3'	876	94°C/ 30 sec; 55^{0}C /30sec; 68^{0}C /30sec; 68^{0}C /11min	Husain *et al*. (2013)
tetA	F: 5' AFGCT ACA TCC TGC TTG CCT TC 3' R: 5' ARCAT AGA TCG CCG TGA AGA GG 3'	210	95^{0}C /3min x30 95^{0}c /1min; 55^{0}C /1min; 72°C/ 1min	Ibrahim et al. (2019)
tetM	F: 5' ACAGAAAGCTTATTATATAAC 3' R: 5' TGGCGTGTCTATGATGTTCAC 3'	171	95^{0}C /3min;x30 95^{0}c/ 1min; 55^{0}C /1min; 72°C/ 1min	Muyzer *et al.,* (1993)
tetK	F: 5' GTA GCG ACA ATA GGT AAT AGT 3' R: 5' GTA GTG ACA ATA AAC CTC CTA 3'	360	95^{0}C /3min;*30 95^{0}C/ 30 sec, 54^{0}C/ 30sec, 72^{0}C/30 sec;	Khoramrooz *et al.,*(2017)
tetO	F: 5' AACTTAGGCATTCTGGCTCAC 3' R: 5' TCCCACTGTTCCATATCGTCA 3'	514	95^{0}C/3min; 95^{0}C/ 30 sec, 54^{0}C/ 30sec 72^{0}C/30 sec:	Khoramrooz *et al.,*(2017)
glmM	F: 5' GAATAAGCTTTTAGGGGTGTTAGGGG 3' R:5' GCTTACTTTCTAACACTAACGCGC 3'	294	94°C/10min 94^{0}C/1min*35 55^{0}C/1min;72^{0}C /1;10min	Rahimi and kheirabadi(2012)
cagA	F: GGCAATGGTGGTCCTGGAGCTAGGC R: GGAAATCTTTAATCTCAGTTCGG	325	94^{0}C /40 sec; 55^{0}C/ 40 sec; 72^{0}C /45 sec; 72^{0}C /6 min.	Mukhopadhyay *et al.,* (2000)

keys:

mecA= cefoxitin resistance gene
ermB= erythromycin resistance gene
nim= metronidazole resistance gene
glmM=helicobacter pylori identification gene
cagA= helicobacter pylori virulence gene
tetA, tetM, tetK, tetO= tetracycline resistance genes

3.2.2 Sample size

The sample size for this study was determined using the formula by Daniel, 1999;

$n = \frac{(Z)^2 * p * (1-p)}{d^2}$ Where n = Sample size of fresh raw cow milk

Z: standard normal distribution at 95% confidence interval= 1.96

P: prevalence from previous study = 0.10% (Uchechi 2016)

D: absolute desired precision at 5% = 0.05

Therefore;

$$n = \frac{(1.96)^2 \times 0.10 \times (1-0.10)}{(0.05)^2} = 138$$

The sample size collected were One hundred and five [105] from lactating cows available in the farm unit as at the three-month period of collection, the difference in the sample size collected was due to the seasonality of the milk production during wet season.

3.2.3 Sampling Method

This study involved convenient method of sampling. Sampling was performed according to the International Dairy Federation guidelines (IDF. 1995; Bauman *et al*., 2018). The cleaning of the herd udder was ensured by using water and the teat of the Cow was disinfected by using a disposable paper towel immersed in a pre-dip iodine solution; in a concentration of one in ten milliliters (1:10) of iodine and water. The cleaning process was performed by the herd handlers, thereafter, a sterile swab stick was used to take sample from the skin teat. The first stream of milk was allowed to run off, thereafter, sample of fresh raw cow milk was collected in sterile universal bottles directly from the cow, the bottle containing the raw milk were then sealed air tight. Containers were marked with source of sample, the date and time of sampling and other relevant information. Also, sterile swab sticks were used to take samples from the handler and some quantity of the water used on the farm

were collected. After collection, the samples of raw cow milk as well as other samples were transported in ice box maintained at a temperature of 4 – 5^0 C and brought to the laboratory. They were preserved in refrigerators and analyzed within one hour.

3.2.4 Research Limitation

Fresh raw cow milk samples were collected from milking cow available in the farm unit with the approval and assistance of the herd handler. This study investigated only the prevailing bacterial isolates obtained from the samples.

3.2.5 Ethical Approval

Ethical clearance for this work was obtained by applying to the Ethical Committee of Ahmadu Bello University ABU for Use of Animal Subjects for Research (ABUCUHSR) and Ethical Committee of National Animal Production Research Institute (NAPRI) Shika, Zaria (Appendix I and II).

3.2.6 Media Preparation

All glassware including petri dishes, test tubes and pipettes used in this analysis were sterilized using a hot oven at 180 $\pm$ 5^0C for at least 1 hour. Each bacteriological medium was prepared from commercially available powder according to the Manufacturer's instructions and sterilized by autoclaving at 121°C for 15 minutes.

3.2.7 Standard Plate Count

The standard plate count was majorly done to check for the microbial load present in the fresh raw milk samples. The bottles of the fresh raw cow milk sample were swabbed with 70% ethanol before opening. Tenfold serial dilutions of samples were carried out in peptone water, then one hundred microliter (0.1 ml) of $1:10^3$ and $1:10^4$ dilutions were plated out using surface plating technique on

Nutrient Agar (NA) for total aerobic bacteria count (TBC) and MacConkey agar (MAC) for total coliform count (TCC) present in the raw milk sample, the plates were then incubated for 24 hours at 35°C. Thereafter, the plates were observed for the presence of discrete colonies and for each dilution, visible colonies that appeared were counted and the average were estimated as colony forming unit per ml (cfu/ml), then the results were recorded.

3.2.8 Methylene Blue Reductase Test (MBRT)

This is a rapid test primarily done to find relative number of bacteria in raw cow milk. One milliliter (1ml) of the methylene blue solution was added to ten milliliters (10ml) of the fresh raw cow milk sample in a sterile covered test tube. It was mixed gently and kept in water bath maintaining a temperature of 37^0C in which observation are made at fixed intervals until the blue disappeared.

3.2.9 Isolation of Bacteria

One hundred microliter (0.1 ml) of $1:10^3$ diluted fresh raw cow milk sample in peptone water was spread each on Mannitol Salt Agar (MSA) and MacConkey agar (MAC) for isolation of *Staphylococcus species* and enteric microorganisms present in the fresh raw cow milk sample respectively. The inoculated plates were then inverted and incubated at 37°C for 24 hours.

The colonies that developed were sub-cultured to further identify pure colony isolates in both MSA and MAC and the plates were incubated at 37^0C for 24hours. Isolates were transferred on nutrient agar slant, incubated and observed for growth at 37°C for 24hours, thereafter, isolates were stored in refrigerator at 4°C pending further studies.

3.2.10 Isolation of *Helicobacter pylori*

Helicobacter pylori grows on different solid media containing blood or blood products with required supplements. Often *H. pylori* grows poorly or not at all on selective media containing antibiotics.

3.2.10.1 Methodology

The procedure of Blanchard and Nedrud (2012) was used in the isolation of *H. Pylori* in this study.

3.2.10.2 Medium Preparation

The standard medium was prepared as described by Blanchard and Nedrud (2012). For five hundred milliliters (500ml) of Blood agar, 21.05g of Blood base agar powder were weighed and dissolved in distilled water by heating. Enough distilled water was added to make five hundred milliliters (500ml). The medium was sterilized at 121^0C at 15 minutes. Upon sterilization, the medium was allowed to cool to 56^0C and 0.015g of vancomycin powder and 35ml of whole sheep blood were added aseptically. The medium was mixed properly and dispensed into sterile petri dish and allowed to solidify.

3.2.10.3 Isolation of *Helicobacter pylori*

The raw cow milk samples from previously incubated peptone water were plated in blood enriched media. The cultured plates were inverted and arranged in anaerobic jar with reduced Oxygen, (5-10%) and water were added to the lower chamber of the anaerobic jar to maintain humidity and to create a microaerophic condition. The inoculated plates were incubated at 37^0Cfor 3-5 days. The isolates were preserved in Brain Heart Infusion Agar slant and stored in microaerophilic condition pending further studies. The isolates were further identified using biochemical analyses and by molecular methods using the *glmM* gene marker.

Also, the presence of *cagA* gene was tested for in the recovered isolates, cagA has been reported as a predictive marker for *Helicobacter pylori* pathogenicity (Feliciano *et al.*,2015).

3.3 Identification of the Isolates

3.3.1 Gram staining

A smear was made on a clean grease free slide and allowed to air dry, the smear was heat fixed and the first stain-Crystal Violet was applied for 60 secs, water was used to flood gently, Iodine was applied as a mordant for 60 secs and flooded gently with water, decolorizer acetone was applied for 10secs and flooded with water, thereafter, the counter stain Carbol fushin was applied for 20-30 secs and flooded gently with water. The stained-glass slide was allowed to dry and viewed under microscope using oil immersion.

3.3.2 Biochemical Tests

Identification of bacterial isolates were carried out following standard methods as reported by Cheesebrough (2006). The test carried out on the isolates include; catalase, oxidase, and coagulase test respectively. Microgen™ Staph-ID and GN ID System were used for further identification of the isolates to the species level.

3.3.2.1 Catalase test

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

3.3.2.2 Oxidase test

Two drops of 1% freshly prepared oxidase reagent (phenylenediamine) was smeared on a filter paper in a clean Petri dish. The test organism was smeared on it with a glass rod. Deep purple appearing within 5-10secs gives a positive result. The absence of deep purple indicates a negative result.

3.3.2.3 Coagulase test

A drop of physiological saline was placed in a clean test tube and a colony was picked from the solid medium and emulsified in saline. A loop full of citrated rabbit plasma was added and mixed using the wire loop. The test tube was held up and tilted back and forth for one minute, agglutination was noted and observations recorded.

3.3.2.4 Urease test

Urea agar was prepared by dissolving 4.2 grams of Urea agar base powder in distilled water by heating, and enough distilled water was added to make 200 milliliters. The medium was sterilized at 121^0c for 15 min and upon sterilization it was allowed to cool to 56^0c and 50 milliliters of sterile 40% Urea solution was added and mixed properly. Thereafter, the medium was dispensed into sterile test tubes aseptically and allowed to solidify in a slanted position. The isolates were inoculated into the slantsand incubated for 3-12 hours and the color change was noted.

3.4 Antimicrobial Susceptibility Test

Discrete colony of isolates on Nutrient Agar plates were emulsified in 5ml sterile physiological saline and turbidity adjusted to 0.5 McFarland standard (approximately a cell density 1.5 x 10^8 cfu/ml) using the modified Kirby-Bauer method. The standardized suspension was inoculated on

prepared Mueller-Hinton agar using sterile cotton swab stick to ensure an even distribution and confluent growth of the isolate. After inoculation, plates were allowed to dry before placing the antimicrobial disks aseptically using sterile forceps. The plates were incubated at 37 °C for 24 hours after which the zone of inhibition was measured in millimeters (mm) and compared against a reference standard and interpreted according Clinical and Laboratory Standard Institute (CLSI 2018).

3.5 Determination of Multiple Antibiotic Resistance (MAR) Index

The MAR Index was determined according to the method of Krumperman (1983) and Paul *et al* (1997). This was calculated by dividing the number of antibiotics to which the isolates were resistant to, by the total number of antibiotics tested.

$$\text{MAR Index} = \frac{\text{Number of antibiotics to which isolates are resistant}}{\text{Total number of antibiotics tested.}}$$

3.6 Molecular Characterization of Resistant Isolates

3.6.1 Bacterial Cell Preparation

Multi antibiotics resistance bacteria were selected based on the resistance categories to three or more antimicrobials. Thirteen (13) isolates were presented for molecular analysis and Pure colonies of antibiotic resistant bacteria isolate from Mueller Hinton Agar were inoculated into 5ml Luria and Bertani (LB) broth and incubated overnight at 37^{o}C for 24h. Bacterial cells were harvested by centrifugation at 4^{o}C, 8000 rpm (6800 ×g) in a microcentrifuge for 2 minutes at room temperature in a microcentrifuge tube, the supernatant was discarded and cells harvested. The step was repeated for higher yield of cells (Lephoto and Gray, 2013).

3.6.2 Genomic DNA Extraction

Genomic DNA extraction was carried out using the method described by Zymo Research Protocol (Lephoto and Gray, 2013). The harvested cell pellets were dislodged and 200µl of deionized water was added and mixed thoroughly by vortexing. Exactly 400µl of the lysis solution were added to the mixture and mixed properly. The mixture was further incubated until the cells were completely lysed and appeared viscous to prevent clogging of the zymo-spin column. Exactly 400µl supernatant were transferred to a zymo-spin™ IV spin filter in collection tube and centrifuged at 7000 rpm for 1 minute. About 1200µl of DNA binding buffers were added to the filtrate in the collection tube from the preceding step.

Exactly 800µlof the mixture from the step above were transferred to a zymo spin IIC Column in a new collection tube and centrifuge at 10000 ×g for 1 minute. The flow through in the collection tube were discarded and the step above repeated. A measure of 200µl DNA pre wash buffer were added to zymo spin column in a new collection tube and centrifuge at 10000 ×g for 1 minute. About 500µl of DNA wash buffer were added to zymo spin column and centrifuge 10000 ×g for 1 minute. The solution was transferred to a clean 1.5ml micro centrifuge tube and 100µl DNA elution buffer added directly to the column matrix and centrifuge at 10000 ×g for 1 minute to elude the DNA (Lephoto and Gray, 2013).

3.6.3 DNA Quantification Measures

To ascertain the concentration and purity of the extracted DNA, the DNA was viewed using the Nano- drop lite and also, the eluent of the DNA was subjected to agarose gel electrophoresis. A combination of 0.8% agarose gel was used to resolve the genomic DNA. This combination was heated in a 250ml beaker flask in a microwave for 2 minutes until the agarose has been dissolved.

The mixture was allowed to cool to room temperature and five (5) µl of ethidium bromide was added as dye to the agarose gel, and mixed. The gel was poured onto a mini horizontal gel electrophoresis tank in which has the casting combs already inserted at the red bands to ensure easy view of the wells. The gel was allowed to set for 30 minutes and after which the genomic DNA was filled into the wells.

The Tris borate EDTA electrophoresis buffer (1x) was then loaded onto the wells of the gel. The mini horizontal electrophoresis gel was set up, covered and connected running from cathode (-) to anode (+). Electrophoresis was carried out at 70mA, 150W, 100 volts for 40 minutes to allow easy separation of the samples based on molecular weight. At the completion of the electrophoresis, the gel was removed from the buffer (TBE) and viewed under a trans-67 illuminator UV light of wavelength 302 nm. The band pattern of the DNA fragments was then photographed with a Polaroid camera and documented using an electrophoresis gel documentation system. The size was compared to the standard DNA molecular weight marker used. The DNA molecular weight marker used was the 1kb ladder.

3.6.4 Polymerase Chain Reaction (PCR) Amplification of Genomic DNA

Amplification of antibiotic resistant genes were carried out using PCR after an external optimization of the reaction to ensure a better amplification. The thin-walled PCR tubes were marked and the following components were added for each isolate for single reaction of 20µl Viz: 6µl of Dream Taq™ PCR master mix was added in the PCR tube, 1.0µl of forward primer, 1.0 of reverse primer, 2.0µl of temple DNA (genomic DNA), nuclease-free water (10µl) were all added in the PCR tube to make up a total volume of 20µl. The samples were spun down and PCR were performed using the thermal cycling conditions as stated by Zymo Research UK (Lephoto and Gray, 2013).

3.6.5 Agarose gel electrophoresis of the PCR products

Ten micro litres (10µl) of the PCR products were loaded into wells of 2% agarose gel containing ethidium bromide. One hundred (100) bp DNA ladder was loaded along sides with the PCR products. Electrophoresis was carried out in Tris borate EDTA buffer at 72mv for 60 minutes until bands were separated. The gel was removed at the completion of electrophoresis and the band pattern viewed using a UV trans-illuminator light of wavelength 302nm. Photographs of bands were taken with polaroid camera and documented using a gel documenting machine (EnduroTM GDS; labnet). The sizes were assessed and estimated from the molecular sizes of the DNA ladder against their migration distance.

3.7 Statistical Analysis

Results are presented using frequencies, percentage, means, standard deviation and range, where appropriate. Statistical Package for Social Science (SPSS) version 20 was used to analyze the data. Chi square test was used to determine associations between categorical variebles.

CHAPTER FOUR

4.1 RESULTS

4.1.1 Distribution of Samples

The overall samples collected from four farm locations in Zaria for this study were One hundred and five (105), from lactating cows available in the farm unit as at the three-month period of collection, this is due to the seasonality of the milk production during wet season. The analysis of the data showed that 74 (70.47%) of the samples were collected from NAPRI; 11 (10.48%) from Zango; 11 (10.48%) from Jamaa lima and 9(8.57%) from Kufena. Samples collected include: fresh raw cow milk; swab from cow teat; swab from herd handler and 500ml of water sample used in cleaning process. Table 4.1 show the overall distribution of samples collected by location and source.

4.2 Percentage of Bacterial Isolates

Of the one hundred and five samples collected, 102(97.1%) had bacteria contaminants (Table 4.2). Seventy-six (76) mixed cultures and twenty-six (26) single cultures were recovered as positive culture while three (3) had no growth. The fresh raw cow milk samples collected appeared fresh, creamy to whitish color with no curd formation observed. The pH readings of the sample milk were between 6.6-6.8 which were within the normal range of standard grade 'A'.

The report of Questionnaire used to evaluate the daily practices held by dairy farmers are presented in Table 4.3. Table 4.4 shows the frequency of occurrence of bacterial isolates from raw cow milk and handlers. The result of total aerobic count, coliform count, and the methylene reductase test carried out on the fresh raw cow milk samples are shown in Appendices III and IV respectively.

The cultural, morphological and biochemical characteristics of the bacteria isolated from this study are shown in Appendix V.

Table 4.1: Total number of Samples collected from various locations

Farm	Fresh raw cow milk	Swab from teat	Swab from handler	Water	Total (%)
Zango	04	04	02	01	**11 (**10.48**)**
Jamaa Lima	04	04	02	01	**11 (**10.48**)**
Kufena	03	03	02	01	**09 (**8.57**)**
NAPRI	31	31	10	02	**74 (**70.47**)**
	42	**42**	**16**	**05**	**105** (100)

Table 4.2: Numbers of Positive Isolates:

Source	Negative culture	Positive culture	
		Mixed	**Single microbe**
Milk	01	34	07
Handler	-	11	05
Teat	02	31	09
Water	-	-	05
Total (%)	**03 (2.9)**	**76 (72.3)**	**26 (24.8)**

Key

Negative culture	No growth recorded
Positive culture	growth recorded
Mixed culture	more than one species of Microbe
Single microbe	one species of Microbe

Table 4.3 Questionnaire used to evaluate the daily practices held by dairy farmers

Parameters	Zango	Jama'a lima	Kufenia	NAPRI
No of Lactating Cows	4	4	4	31
Method of Milking	hand	hand	hand	hand
Pre Dipping (Yes/No)	No	No	Yes	Yes
Product	-	-	Water	Iodine
Composition	-	-	-	1:10
Use of Towel (Yes/No)	No	No	No	Yes
After Dipping (Yes/No)	No	No	No	No
Level of Education	None	None	Primary	Primary
Water Source	Well	River	River	Borehole
Do you Analyse the Water	No	No	No	Yes
Do you sell Raw milk to Consumers	Yes	Yes	Yes	No

Table 4.4: Bacterial Isolates identified from Raw cow milk and handlers

S/N	Bacteria isolated	No.of positive isolates
1	*Staphylococcus aureus*	10
2	*Staphylococcus xylosus*	1
3	*Staphylococcus hyicus*	4
4	*Staphylococcus chromogens*	2
5	*Staphylococcus intermedius*	1
6	*Micrococcus leutus*	2
7	*Pantoea agglomerance*	3
8	*Citrobacter freundi*	1
9	*Acinetobacter iwoffii*	15
10	*Citrobacter youngae*	1
11	*Proteus vulgaris*	7
12	*Moraxella spp*	7
13	*Pseudomonas aeruginosa*	3
14	*Aeromonas caviae*	1
15	*Aeromonas hydrophila*	3
16	*Pasteurella multocida*	1
17	*Klebsiella oxytoca*	1
18	*Escherichia coli*	3
19	*Yersinia enterolitica*	1
20	*Weeksela zoohelcum*	1
21	*S.choleraesus*	1
22	*Providencia stuarti*	2
23	*Hafnia alveri*	2
24	*Klebsiella pneumonia*	1
25	*Proteus mirabilis*	1
26	*Vibrio parahaemolyticus*	1
27	*Burkholderia pseudomallei*	1
28	*Plesiomonas shigelloides*	1
29	*Helicobacter pylori*	12
	Total	92

4.3 Mean Total Viable Count of Isolates

The mean total aerobic bacteria count from fresh raw cow milk samples collected from four farm locations; Zango, Jama'a limaa, Kufenia and NAPRI are as shown in Table 4.5.

Raw milk sampled from Zango and Jama'a lima farms have higher numbers of *Staphylococci species* (Table 4.6 and Table 4.7). *Proteus vulgaris* were the most recovered isolates from milk sampled from Kufena farm and was also recovered from cow teat and handler swabs (Table 4.8).

Raw milk sampled from NAPRI had higher numbers of *occurrence of Acinetobacter iwoffi* among the Gram-negative isolates and higher numbers of occurrence of *Staphylococci* species among the Gram-positive isolates (Table 4.9). *A. iwoffi* was also recovered from cow teat and handler swabs (Table 4.9). There was no significant association between sampling location and positive bacterial recovery, however, milk samples from NAPRI showed higher bacterial recovery rates compared to other farms, while teat swab samples from Zango farm were more likely to return positive for bacterial growth ($p=0.270$).

Table 4.5: Mean Total Viable Count of Isolates from Fresh raw cow milk

Farm	TABC ($x10^4$cfu/ml)	TCC ($x10^4$cfu/ml)
Zango	1.13±0.64	0.58±0.70
Sabo	2.43±1.57	0.98±0.83
Kufena	0.8±0.79	0.87±0.49
NAPRI	2.93±2.88	1.16±1.1
Overall Mean	2.56±0.40	1.06±0.16
Std. Deviation	2.62	1.00

Key:
TBC Total Aerobic Bacteria count
TCC Total Coliform count

Table 4.6: Bacteria Isolates obtained from Zango Farm

Location	Organism	Source				Total (%)
		Milk	Teat	Handler	Water	
	Gram Positive	**(02)**	**(02)**	**(0)**	**(0)**	**(04)**
	Staphylococcus aureus	01	-	-	-	01 (25)
	Staphylococcus chromogenes	-	01	-	-	01 (25)
	Staphylococcus hyicus	01	-	-	-	01 (25)
	Staphylococcus xylosus	-	01	-	-	01 (25)
	Gram Negative	**(01)**	**(02)**	**(01)**	**(01)**	**(05)**
	Acinetobacter iwoffi	-	01	-	01	02 (40)
	Klebsiella pneumoniae	-	-	01	-	01 (20)
	Pasteurella multocida	01	-	-	-	01 (20)
	Providencia stuartii	-	01	-	-	01 (20)

Table 4.73: Bacteria Isolates obtained from Jama'a lima Farm

Location	Organism	Source				Total (%)
		Milk	Teat	Handler	Water	
Sabo	**Gram Positive**	**(01)**	**(0)**	**(0)**	**(0)**	**(02)**
	Staphylococcus chromogenes	01	-	-	-	01 (50)
	Staphylococcus hyicus	01	-	-	-	01 (50)
	Gram Negative	**(01)**	**(01)**	**(01)**	**(01)**	**(06)**
	Citrobacter youngae	-	-	-	01	01 (16.7)
	Escherichia coli	-	-	01	-	01 (16.7)
	Flavobacterium odoratum	-	01	-	-	01 (16.7)
	Klebsiella oxytoca	-	01	-	-	01 (16.7)
	Pseudomonas aeruginosa	01	-	01	-	02 (33.3)

Table 4.84: Bacteria Isolates obtained from Kufenia Farm

Location	Organism	Source				Total (%)
		Milk	Teat	Handler	Water	
Kufena	**Gram Positive**	**(01)**	**(01)**	**(01)**	**(0)**	**(03)**
	Staphylococcus aureus	01	01	-	-	02 (66.7)
	Staphylococcus intermedius	-	-	01	-	01 (33.3)
	Gram Negative	**(04)**	**(03)**	**(02)**	**(01)**	**(10)**
	Acinetobacter iwoffi	-	01	-	-	01 (10)
	Aeromonas caviae	01	-	-	-	01 (10)
	Citrobacter freundi	01	-	-	-	01 (10)
	Escherichia coli	-	-	-	01	01 (10)
	Proteus vulgaris	02	02	01	-	05 (50)
	Yersinia enterocolitica	-	-	01	-	01 (10)

Table 4.95: Bacteria Isolates obtained from NAPRI

Location	Organism	Source				Total (%)
		Milk	Teat	Handler	Water	
Giwa	**Gram Positive**	**(11)**	**(0)**	**(0)**	**(0)**	**(11)**
1.	*Micrococcus luteus*	02	-	-	-	02 (18.2)
2.	*Staphylococcus aureus*	07	-	-	-	07 (63.6)
3.	*Staphylococcus hyicus*	02	-	-	-	02 (18.2)
	Gram Negative	**(24)**	**(08)**	**(06)**	**(01)**	**(39)**
1.	*Acinetobacter iwoffi*	08	03	01	-	12 (30.8)
2.	*Aeromonas hydrophila*	03	-	-	-	03 (7.6)
3.	*Burkholderia pseudomallei*	01	-	-	-	01 (2.6)
4.	*Escherichia coli*	01	-	-	-	01 (2.6)
5.	*Hafnia alvei*	01	01	-	-	02 (5.**1**)
6.	*Moraxella species*	04	01	01	01	07 (17.9)
7.	*Pantoea agglomerans*	02	-	01	-	03 (7.6)
8.	*Plesiomonas shigelloides*	01	-	-	-	01 (2.6)
9.	*Proteus mirabilis*	-	-	01	-	01 (2.6)
10.	*Proteus vulgaris*	-	01	01	-	02 (5.**1**)
11.	*Providencia stuartii*	-	01	-	-	01 (2.6)
12.	*Pseudomonas aeruginosa*	02	-	-	-	02 (5.**1**)
13.	*S. choleraesus*	-	-	01	-	01 (2.6)
14.	*Vibrio parahaemolyticus*	01	-	-	-	01 (2.6)
15.	*Weeksela zoohelcum*	-	01	-	-	01 (2.6)

4.4 Cultural and Biochemical characteristics of *Helicobacter pylori*

Twelve of the isolates showed positive cultural characteristic of the *H. pylori* on the antibiotics supplemented blood agar. They were observed to be translucent with hemolysis formed ranging from alpha to gamma (Fig 4.1).

The result of the morphological characteristics and the biochemical test carried out on the *Helicobacter pylori* isolates are presented in Appendix VI.

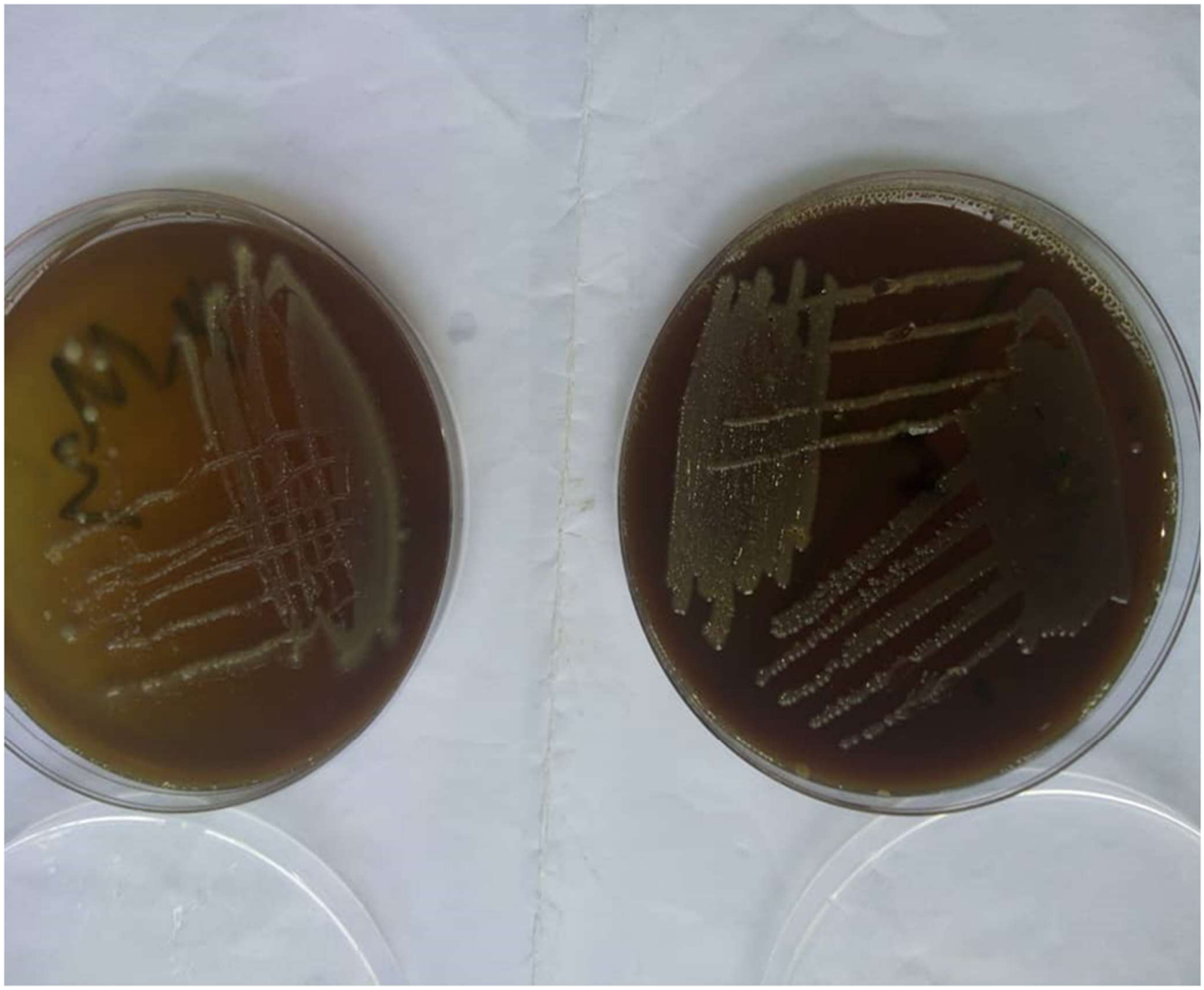

Plate 4.1: Cultural characteristic of *Helicobacter pylori* Isolate
Source: This study

4.5 Antimicrobial Susceptibility of Identified Isolates

Most of the *Staphylococcus* species and *Micrococcus luteus* isolates were methicillin resistant (85%) and showed considerable resistance to tetracycline (75%) and amoxicillin-clavulanic acid (85%) (Table 4.10). Chloramphenicol was the most active antimicrobial agent against the *Staphylococcal* species and *Micrococcus luteus* identified in this study (90%), followed by Ciprofloxacin (85%), Gentamicin (80%) and Erythromycin (75%). The level of resistance was categorized based on the International Expert Proposal on Interim Standard Definitions for acquired resistance (Magiorakos *et al.,* 2012) as Multidrug resistant (MDR): non-susceptible to ≥1 agent in ≥3 antimicrobial categories, extensively drug resistant (XDR): non-susceptible to ≥1 agent in all but ≥2 antimicrobial categories, Pandrug resistant (PDR): non-susceptible to all antimicrobial agents. The Antimicrobial Resistance Percentage (Table 4.11) shows the level of resistance to the tested categories antimicrobials. Eighty percent of *S. aureus* isolates were multidrug resistant (Table 4. 12).

Acinetobacter iwoffii and other members of the Enterobacteriaceae isolated in this study were resistant to metronidazole (96%) and ceftazidime acid (89%) and they also showed considerable resistance to erythromycin (71.74%) and tetracycline (68.75%) (Table 4.13). The susceptibility of the Enterobacteriaceae to the tested antimicrobials showed that 94.34% were susceptible to Gentamicin, 80.85% to Chloramphenicol and 77.08% to Ciprofloxacin.

The percentage of antimicrobial resistance (Table 4.14) showed the level of resistance to the tested categories of antimicrobials. *Acinetobacter iwoffii* were highly resistant to three antimicrobials in about three different classes, *Pseudomonas aeruginosa* isolates also showed major resistant to seven antimicrobials in about four different classes. Seventy-one point four (71.4%) of *Acinetobacter iwoffii* isolates were multidrug resistant, while *Pseudomonas aeruginosa* were extensively drug resistant (Table 4.15).

Table 4.10: Susceptibility of the *Staphylococcus species* and *Micrococcus luteus* to different groups of Antimicrobial

Antimicrobials	Disc Content(ug)	NT	Resistance (%)	Susceptible (%)
Cefoxitin	30	20	17(85)	3(15)
Amoxi-Clavulanic	30	20	17(85)	17(85)
Ampicillin	10	20	13(65)	7(35)
Tetracycline	30	20	15(75)	5(25)
Ciprofloxacin	5	20	3(15)	17(85)
Gentamicin	10	20	4(20)	16(80
Erythromycin	15	20	5(25)	15(75)
Chloramphenicol	30	19	1(5)	18(90)

NB: NT indicates number of tested Isolates

Table 4.11: Percentage of Antimicrobial Resistance *Staphylococcus species* and *Micrococcus luteus*

S/N	Bacteria isolates	Antimicrobial Resistance (%)							
		FOX	**TET**	**CIP**	**AMP**	**CN**	**ERY**	**AMC**	**CHL**
1.	*Micrococcus luteus* (n=02)	50	100	0	50	0	50	50	0
2.	*Staphylococcus aureus* (n=10)	80	70	0	60	10	20	100	10
3.	*Staphylococcus chromogens* (n=02)	100	50	100	100	50	50	100	0
4.	*Staphylococcus hyicus* (n=04)	100	75	0	75	25	25	75	0
5.	*Staphylococcus intermedius* (n=01)	100	100	0	0	100	0	100	0
6.	*Staphylococcus xylosus* (n=01)	100	100	100	100	0	0	0	0

FOX = cefoxitin, ERY = Erythromycin, AMP = Ampicillin, TET = Tetracycline, AMC = Amoxicillin Clavulanic acid, CN = Gentamicin, CIP = Ciprofloxacin, CHL = Chloramphenicol

Table 4.62: Classification of Multiantibiotic drug Resistance *Staphylococcus species* and *Micrococcus luteus Isolates*

S/N	Bacteria isolates	Antimicrobial Resistance (%)			
		PDR	XDR	MDR	nMDR
1.	*Micrococcus luteus* (n=02)	0	0	50	50
2.	*Staphylococcus aureus* (n=10)	0	10	80	10
3.	*Staphylococcus chromogens* (n=02)	0	50	50	0
4.	*Staphylococcus hyicus* (n=04)	0	0	75	25
5.	*Staphylococcus intermedius* (n=01)	0	0	100	0
6.	*Staphylococcus xylosus* (n=01)	0	0	100	0

Key:

PDR: Pandrug resistant (resistant to All classes of antimicrobials)

XDR: Extensively drug resistant (resistant to one antimicrobial in all but two classes)

MDR: Multidrug resistant (resistant to one antimicrobial in three or more classes)

nMDR: non Multidrug resistant

Table 4.13: Susceptibility of the *Enterobacteriaceae* to different groups of Antimicrobial

Antimicrobials	Disc content (ug)	NT	Resistance(%	Susceptible (%)
Ceftazidime	10	53	47(88.68)	6(11.32)
Tetracycline	30	48	33(68.75)	15(31.25)
Ciprofloxacin	5	48	11(22.92)	37(77.08)
AmoxiClavulanic	30	52	35(67.31)	17(32.69)
Ampicillin	10	36	19(52.78)	17(47.22)
Gentamicin	10	53	3(5.66)	50(94.34)
Erythromycin	15	46	33(71.74)	13(28.26)
Chloramphenicol	30	47	9(19.15)	38(80.85)
Metronidazole	10	52	52(96)	-

NB: NT indicates number of tested Isolates

Table 4.14: Antimicrobial Resistance of Enteric Bacteria Isolates

S/N	Bacteria isolates	Antimicrobial Resistance (%)								
		CAZ	**TET**	**CIP**	**AMP**	**CN**	**ERY**	**AMC**	**CHL**	**MTZ**
1.	*Acinetobacter iwoffii* (n=14)	85.7	35.7	7.1	42.9	7.1	42.9	78.6	14.3	100
2.	*Aeromonas hydrophila* (n=03)	100	33.3	0	66.7	0	100	100	33.3	100
3.	*Burkholderia pseudomallei* (n=01)	0	100	100	-	0	100	100	0	100
4.	*Citrobacter freundi* (n=01)	100	0	0	0	0	100	0	0	100
5.	*Citrobacter youngae* (n=01)	100	100	100	100	100	100	100	0	100
6.	*Escherichia coli* (n=02)	100	0	0	50	0	0	50	0	100
7.	*Hafnia alvei* (n=02)	0	50	50	0	0	50	50	0	50
8.	*Klebsiella oxytoca* (n=01)	100	100	0	100	0	100	100	0	100
9.	*Klebsiella pneumoniae* (n=01)	100	0	0	100	0	100	100	0	100
10.	*Moraxella specie* (n=06)	100	100	0	50	16.7	33.3	50	16.7	100
11.	*Pantoea agglomerans* (n=03)	33.3	66.7	0	0	0	66.7	33.3	0	66.7
12.	*Pasteurella multocida* (n=01)	100	100	0	100	0	100	100	100	100
13.	*Plesiomonas shigelloides* (n=01)	100	0	0	100	0	100	100	100	100
14.	*Proteus vulgaris* (n=07)	100	71.4	57.1	42.9	0	85.7	42.9	0	100
15.	*Proteus mirabilis* (n=01)	100	100	0	100	0	100	0	0	100

Table 4.14: Antimicrobial Resistance of Enteric Bacteria Isolates Contd'

S/N	Bacteria isolates	Antimicrobial Resistance (%)								
		CAZ	**TET**	**CIP**	**AMP**	**CN**	**ERY**	**AMC**	**CHL**	**MTZ**
16.	***Providencia stuarti* (n=02)**	**100**	**50**	**50**	**50**	**0**	**50**	**0**	**0**	**100**
17.	*Pseudomonas aeruginosa* (n=03)	100	100	33.3	66.7	0	100	100	66.7	100
18.	*S. choleraesus*	100	100	0	100	0	0	100	100	100
19.	*Vibrio parahaemolyticus*	100	100	0	100	0	0	100	0	100
20.	*Weeksela zoohelcum*	100	0	0	0	0	0	0	0	100
21.	*Yersinia enterolitica*	100	100	100	100		100	100	0	100

CAZ = Ceftazidime, AMP = Ampicillin, AMC = Amoxicillin Clavulanic acid, TET = Tetracycline,

CN = Gentamicin, CHL = Chloramphenicol, CIP = Ciprofloxacin, ERY = Erythromycin, MTZ= Metronidazole

Table 4.15: Classification of Multiantibiotic drug Resistant Enteric Bacteria Isolates

S/N	Bacteria isolates	(%)			
		PDR	XDR	MDR	nMDR
1.	*Acinetobacter iwoffii* (n=14)	0	21.4	71.4	7.1
2.	*Aeromonas hydrophila* (n=03)	0	66.7	33.3	0
3.	*Burkholderia pseudomallei* (n=01)	0	0	100	0
4.	*Citrobacter freundi* (n=01)	0	0	100	0
5.	*Citrobacter youngae* (n=01)	0	100	0	0
6.	*Escherichia coli* (n=02)	0	0	50	50
7.	*Hafnia alvei* (n=02)	0	0	50	50
8.	*Klebsiella oxytoca* (n=01)	0	100	0	0
9.	*Klebsiella pneumoniae* (n=01)	0	0	100	0
10.	*Moraxella specie* (n=06)	0	33.3	66.7	0
11.	*Pantoea agglomerans* (n=03)	0	0	66.7	33.3
12.	*Pasteurella multocida* (n=01)	0	100	0	0
13.	*Plesiomonas shigelloides* (n=01)	0	100	0	0
14.	*Proteus vulgaris* (n=07)	0	42.9	57.1	0
15.	*Proteus mirabilis* (n=01)	0	0	100	0
16.	*Providencia stuarti* (n=02)	0	50	0	50
17.	*Pseudomonas aeruginosa* (n=03)	0	100	0	0
18.	*S. choleraesus*	0	100	0	0
19.	*Vibrio parahaemolyticus*	0	0	100	0
20.	*Weeksela zoohelcum*	0	0	0	100
21.	*Yersinia enterolitica*	0	100	0	0

Key:

PDR: Pandrug resistant (resistant to All classes of antimicrobials)

XDR: Extensively drug resistant (resistant to one antimicrobial in all but two classes)

MDR: Multidrug resistant (resistant to one antimicrobial in three or more classes)

nMDR: non Multidrug resistant

Overall, 53.8% of the isolates were multidrug resistant. There was no significant association between sample source and multi-resistance phenotype, however, isolates from teat swabs were more likely to be non-multidrug resistant (p= 0.555).

There was no significant association between sampling location and multi-resistance phenotype, however, isolates from Zango farm were more likely to be non-multidrug resistant compared to strains from Sabo farm, most of which were extensively drug resistant (p= 0.145).

In this study, the percentage of multiple antibiotic resistance index (MARI) of greater than or equal to 0.3 was observed to be 90% for *Staphylococcus species* (Table 4.16) and 92% for Enterobacteriaceae (Table 4.17).

Table 4.76: Multiple Antibiotic Resistance Index (MARI) of *Staphylococcus* species and *Micrococcus luteus* Isolates

MARI	Distribution	Percentage (%)	
0.1	02	10	
0.3	01	5	
0.4	06	30	
0.5	04	20	90%
0.6	05	25	
0.8	02	10	

Table 4.17: Multiple Antibiotic Resistance Index (MARI) of Enteric Bacteria Isolates

MARI	Distribution	Percentage (%)	
0.2	04	8	
0.3	08	16	
0.4	05	10	
0.5	04	8	
0.6	11	22	
0.7	07	14	92%
0.8	08	16	
0.9	03	6	

4.6 Polymerase Chain Reaction (PCR) Amplification of Target Genes

Electrophoretogram of extracted DNA of thirteen isolates is displayed in plate 4.2. The amplification of *nim* (458bp) and *ermB* (639bp) from isolates obtained from fresh raw cow milk and handler are shown in plate 4.3 and 4.4 respectively. The *nim* amplified in one (1) out of the four (4) isolates; *Proteus mirabilis* from handler in NAPRI.

The *ermB* amplified in three (3) isolates; *Pseudomonas aeruginosa* from raw milk in NAPRI, *Proteus vulgaris* from raw milk in Kufena farm and *S. choleraesus* from handler in NAPRI (Plate 4.4). There was no amplification of MecA, tetK, tetL and tetM from the isolates obtained from fresh raw cow milk and handlers (Table 4.18).

The *tetA* (210bp) result is presented in plate 4.5., Similary, *tetA* (210bp) was amplified in both *Pseudomonas aeruginosa* from raw milk in NAPRI and *Proteus vulgaris* from raw milk in Kufena farm (Table 4.19).

Amplification of *cagA* genes is shown in Plate 4.6. The was no amplification of *glmM* (294bp) to identify *Helicobacter pylori*, while the virulence marker of *Helicobacter pylori cagA* (325bp), amplified in three (3) out of the five isolates; they were *Helicobacter pylori* recovered from raw cow milk in Kufenia and NAPRI (Table 4.20).

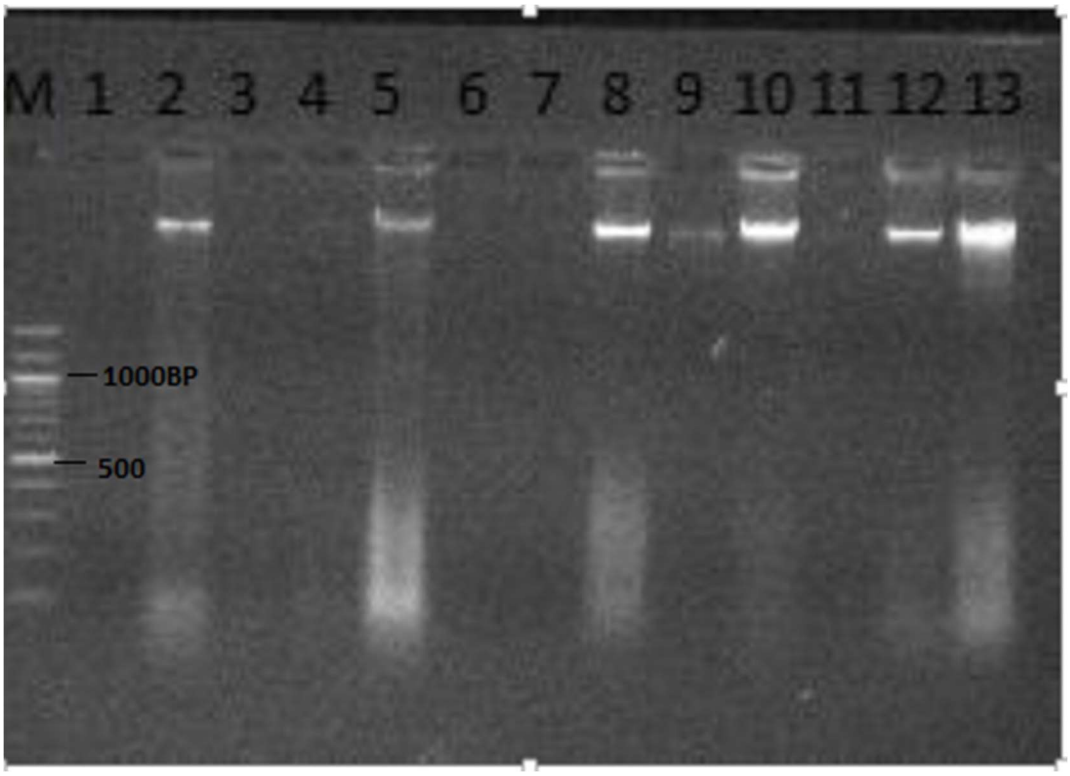

Plate 4.2: Electrograph of extracted DNA

Key:

M= 100 bp ladder; lane 1-13= bacteria DNA

1= GH2: *Proteus mirabilis* recovered from handler

2= GM2: *Pantoea agglomerans* recovered from fresh raw cow milk

3= GM13: *Staphylococcus hyicus* recovered from fresh raw cow milk

4= SM1: Staphylococcus hyicus recovered from fresh raw cow milk

5=GM27: *Aeromonas hydrophila* recovered from fresh raw cow milk

6= GM17: Staphylococcus aureus recovered from fresh raw cow milk

7= GM3: *Hafnia alveri* recovered from fresh raw cow milk

8= KM2: *Helicobacter pylori* recovered from fresh raw cow milk

9= GM25: *Helicobacter pylori* recovered from fresh raw cow milk

10= GM2: *Helicobacter pylori* recovered from fresh raw cow milk

11= GM15: *Helicobacter pylori* recovered from fresh raw cow milk

12= KM3: *Helicobacter pylori* recovered from fresh raw cow milk

13= GM23: *Helicobacter pylori* recovered from fresh raw cow milk

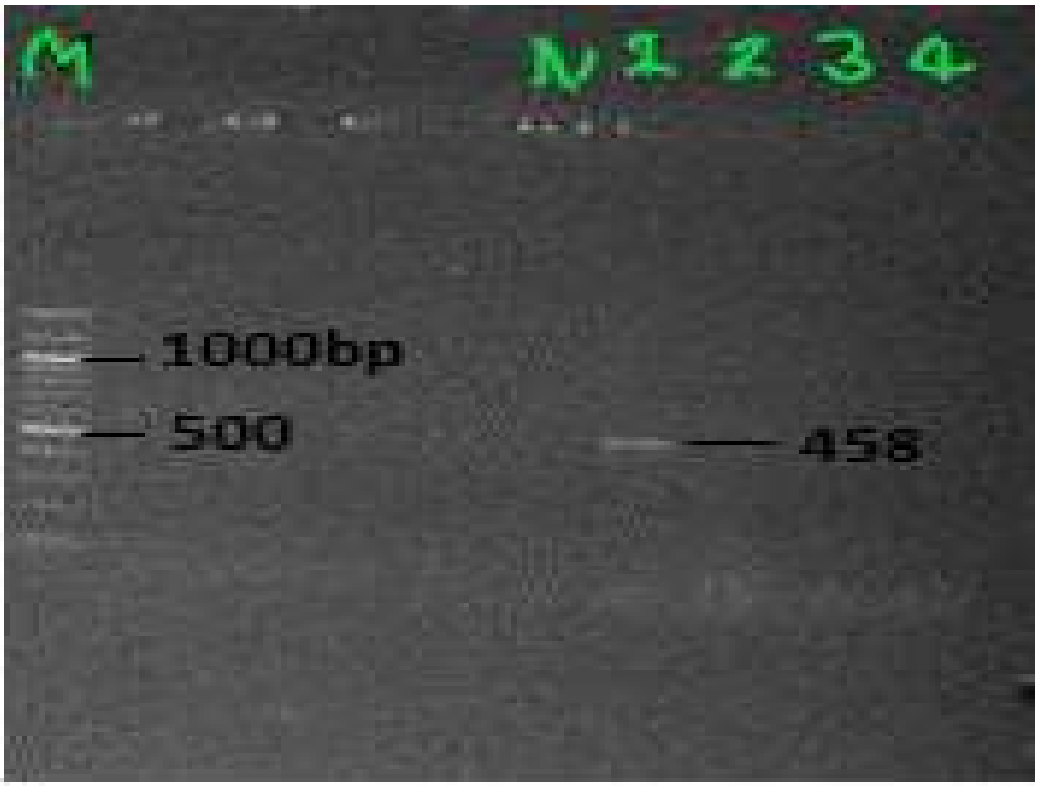

Plate 4.3: Electrograph of *nim* (458bp) isolate recovered from fresh milk and handler sample in Zaria

Key:

M= 100 bp ladder;

lane 1= GH2 ; *Proteus mirabilis* recovered from handler

lane 2= GM2 ; *Pantoea agglomerans* recovered from fresh raw cow milk

lane 3= GM27; *Aeromonas hydrophila* recovered from fresh raw cow milk

lane 4= GM3; *Hafnia alveri* recovered from fresh raw cow milk

lane N= negative control.

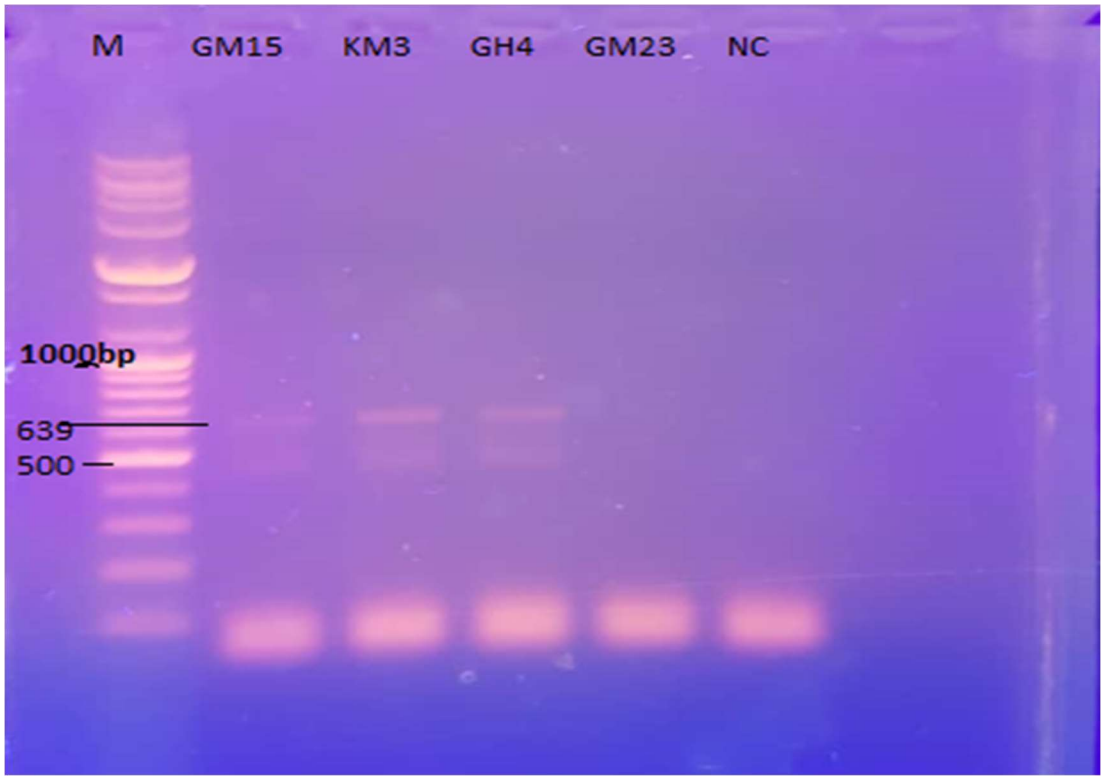

Plate 4.4: Electrograph of *ermB* (639bp) from isolates recovered from fresh milk and handler in Zaria.

Key:

M= molecular ladder;

Lane 1:GM15= *Pseudomonas aeruginosa* recovered from fresh raw cow milk

Lane 2:KM3= *Proteus vulgaris* recovered from fresh raw cow milk

Lane 3:GH4= *S.choleraesus* recovered from handler

Lane 4:GM23= *Acinetobacter iwoffi* recovered from fresh raw cow milk

Lane 5:NC= negative control

Table 4.18: Detection of *mecA, tetK, tetL, tetM* and *nim* in bacteria isolates

S/N	Organism	Source	*mecA*	*tetK*	*tetL*	*tetM*	*nim* (458bp)
1.	*Proteus mirabilis* GH2	Handler	-	-	-	-	+
2.	*Pantoea agglomerans* GM2	Raw cow milk	-	-	-	-	-
3.	*Staphylococcus hyicus* GM13	Raw cow milk	-	-	-	-	NT
4.	*Staphylococcus hyicus* SM1	Raw cow milk	-	-	-	-	NT
5.	*Aeromonas hydrophila* GM27	Raw cow milk	-	-	-	-	-
6.	*Staphylococcus aureus* GM17	Raw cow milk	-	-	-	-	NT
7.	*Hafnia alvei* GM3	Raw cow milk	-	-	-	-	-

Key:

GH2, GM2, GM13, SM1, GM27, GM17 are sample codes

NT not tested; - = no amplification

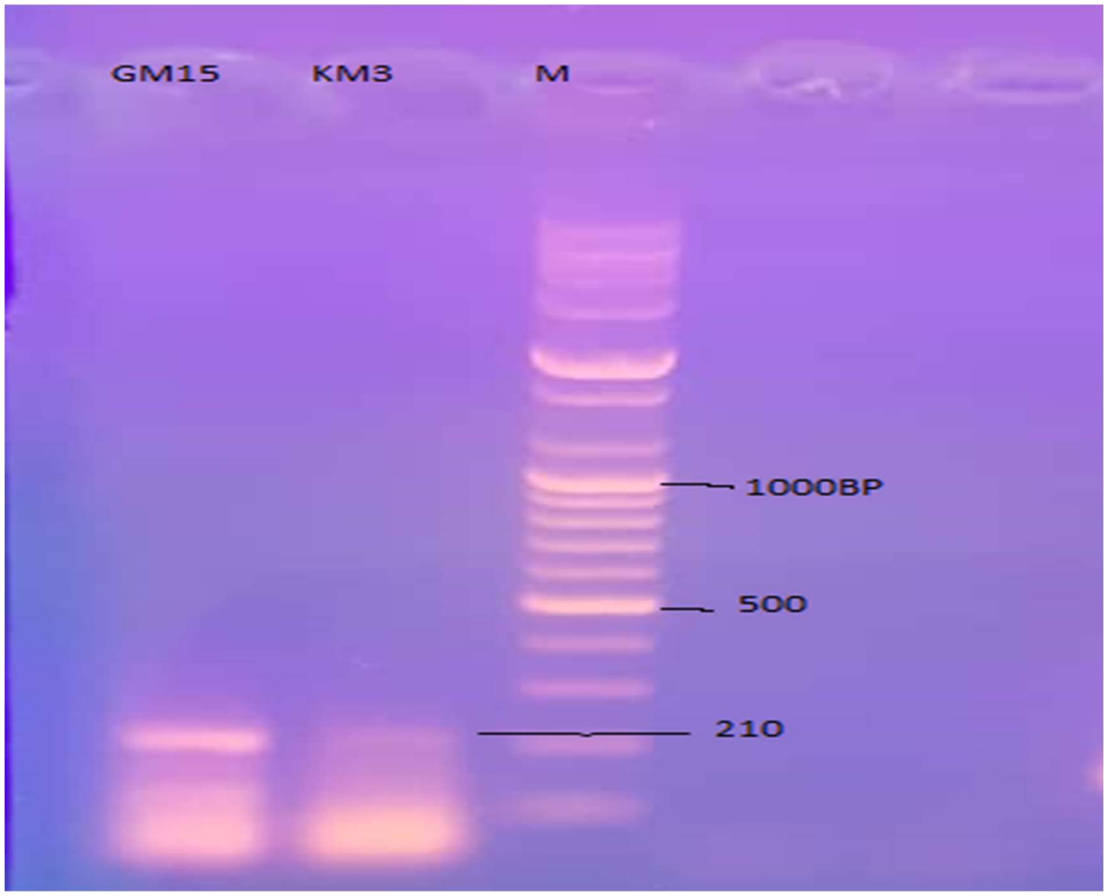

Plate 4.5: Electrograph of *tetA* (210bp) recovered from and *Pseudomonas aeruginosa* and *Proteus vulgaris* isolated from fresh raw cow milk in Zaria.

Key:

M= molecular ladder;

Lane 1: GM15= *Pseudomonas aeruginosa* recovered from fresh raw cow milk

Lane 2: KM3= *Proteus vulgaris* recovered from fresh raw cow milk

Table 4.19: Detection of *ermB* and *tetA* genes in Enterobacteriaceae isolates

S/N	Organism	Source	*ermB* (639bp)	*tetA* (210bp)
1.	*Pseudomonas aeruginosa* GM15	Fresh raw cow milk	+	+
2.	*Proteus vulgaris* KM3	Fresh raw cow milk	+	+
3.	*S. choleraesus* GH4	Handler	+	NT
4.	*Acinetobacter iwoffi* GM23	Fresh raw cow milk	-	NT

Key:

GM15; fresh raw cow milk from NAPRI

KM3; fresh raw cow milk from Kufena farm

GH4; handler from NAPRI

GM23; fresh raw cow milk from NAPRI

Table 4.20: Detection of *cagA* and *glmM* genes in *Helicobacter pylori* isolates

S/N	Organism	Source	*glmM* (294bp)	*cagA* (325bp)
1.	*Helicobacter pylori* KM2	Fresh raw cow milk	-	+
2.	*Helicobacter pylori* GM25	Fresh raw cow milk	-	-
3.	*Helicobacter pylori* GM2	Fresh raw cow milk	-	+
4.	*Helicobacter pylori* GM15	Fresh raw cow milk	-	-
5.	*Helicobacter pylori* KM3	Fresh raw cow milk	-	+

Key

KM2: fresh raw cow milk from Kufenia farm

GM25: fresh raw cow milk from NAPRI

GM2: fresh raw cow milk from NAPRI

GM15: fresh raw cow milk from NAPRI

KM3: fresh raw cow milk from Kufena farm

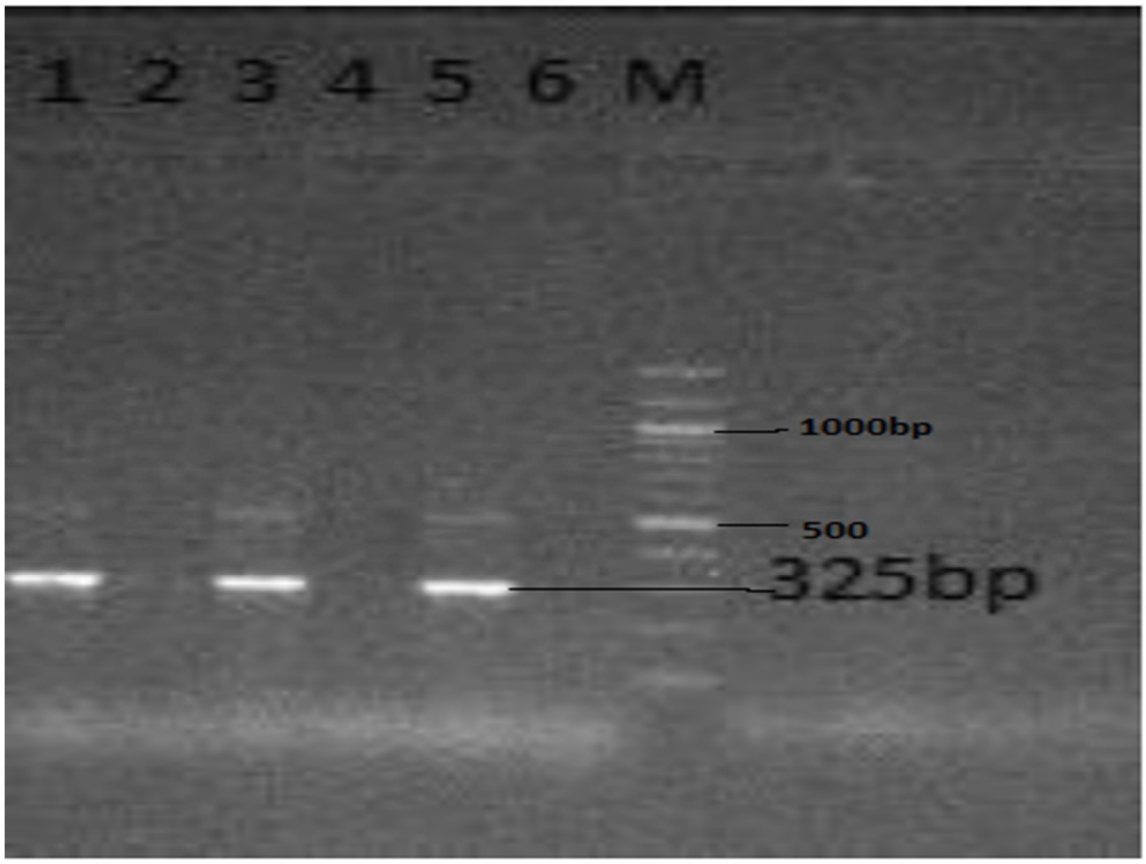

Plate 4.6: Electrograph of *cagA* (325bp) recovered from *Helicobacter pylori* isolates

Key:

M= 100 bp ladder;

lane 1= KM2 recovered from fresh raw cow milk

lane 2= GM25 recovered from fresh raw cow milk

lane 3= GM2 recovered from fresh raw cow milk

lane 4= GM15 recovered from fresh raw cow milk

lane 5= KM3 recovered from fresh raw cow milk

lane 6= negative control

CHAPTER FIVE

5.1 DISCUSSION

In this study, bacteria isolated from raw cow milk samples and handlers were mostly members of the Enterobacteriaceae family, which are common inhabitants of the intestinal tract of various domestic animals. They are commonly found in cow dung, water and are also regarded as environmental pathogens which could easily be introduced to our food and water including raw milk during milking processes. The bacteria isolates recovered from herd handlers, teat of the cow and the water used in the cleaning process give an indication of possible contaminating sources of the fresh raw cow milk; contaminations could have been from improper teat disinfection or by inadequate hygiene practiced by the herd handlers or the use of untreated water that is being used in the cleaning process. The handler's negligence and their low level of education in proper udder preparation were strong indications for the high level of microbial load of the raw milk analyzed in this study. None of them made use of detergent to wash the Udder and about 70% of the farmers used a single pre-dip solution in cleaning about 4-5 cows with a towel. The water source used in the three farms visited was from the underground well and no prior treatment done for purification before use. Also, the handlers did not make use of detergent in washing their hand before contact with the cows.

The total aerobic bacteria count (TABC) and total coliform count (TCC) observed in this study were $2.56\pm0.40x10^4$ cfu/ml and $1.06\pm0.16x10^4$ cfu/ml respectively. The TABC and TCC are relatively high when compared to the standard limits given by Food and Drug Association (FDA) pasteurized milk ordinance for grade A use (PMO, 2009) of <10- 10^5cfu/ml for Total Viable Bacteria Count (TVBC) and ≤ 10 cfu/ml for Total coliform count respectively. This could be attributed majorly to several ineffective practices on the farm which resulted in contamination of the raw milk. Although,

the TBC and TCC from this study are low when compared with report by Oladipo *et al*. (2016), with TBC and TEC of 0.2- 4.2x10^6 cfu/ml and 0.8- 2.6x10^6 cfu/ml respectively in Ogbomosho Nigeria and also lower compared to report by Al-bajaly *et al*. (2021) of Total Viable Bacteria Count (TVBC) of 140.43±25.19X10^6 in Iraq.

Acinetobacter species are the most prevalent among the Gram-negative bacteria isolated in this study and extensively resistant to many approved antimicrobial agents. They are psychotropic and found in soil and water, as a normal flora of animals and human skin. This may explain their high occurrence in the raw milk samples examined in this study. *Acinetobacter spp.* are considered as one of the major causes of nosocomial infections worldwide (Almasaudi, 2018). Investigations by Saad *et al.* (2018) and Hoque *et al.* (2019) reported the isolation of higher number of *Acinetobacter species* from raw milk and milk products which is in agreement to the findings in this study.

The antimicrobial resistance pattern observed among the identified Enterobacteriaceae in this study against amoxicillin- clavulanic acid (6 7.31%), tetracycline (68.75%), erythromycin (71.74%), ceftazidime (88.68%) and metronidazole (96%) agree with the antimicrobial resistance to tetracycline and erythromycin respectively reported by Mbuk *et al*. (2016) in Kaduna, Nigeria and resistance to erythromycin (85.71%) reported by Tamba *et al.,* (2016) in Zaria. High susceptibility was observed to gentamicin (94.34%), chloramphenicol (80.85%), and ciprofloxacin (77.08%) among the identified Enterobacteriaceae in this study. This is in close relation to what Tamba *et al*. (2016) reported for gentamicin and ciprofloxacin (100%) and chloramphenicol (93%). It is also similar to what Oladipo *et al*. (2016) in Ogbomosho, Nigeria reported for both gentamicin and ciprofloxacin in all the tested isolates of coliform from raw milk. The pattern of susceptibility and resistance exhibited in the present study may be due to prolonged and indiscriminate usage and

prescription of some particular antibiotics which often leads to possible resistance development in animals (Kwaga, 2012; Sharma, 2014).

Most of the *Staphylococcus species* isolated from this study were methicillin resistant (85%) and showed considerable resistance to tetracycline (75%) and amoxicillin-clavulanic acid (85%). Chloramphenicol was the most active antimicrobial agent against the Staphylococcal species identified in this study with 90%, followed by ciprofloxacin (85%), gentamicin (80%) and erythromycin (75%). The findings from this study are similar to what was reported by Shamila-Syuhada *et al*. (2016) from a small-scale dairy in Penang, Malaysia in which *Staphylococcus* species isolated from milk samples were 100% susceptible to ciprofloxacin and gentamicin and chloramphenicol (50%). Chloramphenicol has generally been withdrawn from use in most countries due to the damage it can introduce to the individual especially to the bone marrow (Mark *et al.*, 2016). Susceptibility observed to chloramphenicol in this study shows relatively it will be one of the most reliable antibiotics if reintroduced to the populace since it has been used for many years. Resistance was however reported to tetracycline and erythromycin. Similar result was reported by Okpo *et al*. (2017) in some parts of Kaduna State, Nigeria, where isolated *Staphylococcus aureus* from fresh milk and milk products had susceptibility to gentamicin and ciprofloxacin (100%), chloramphenicol (94.4%), while resistance was reported to amoxicillin-clavulanic acid (50%), cefoxitin (71.4%) and tetracycline (64.3%). Also, Abd-El halem *et al*. (2019) in Alexandra Egypt reported that all tested *Staphylococcus* species isolates were susceptible to ciprofloxacin. Aliyu *et al*. (2019), in a study on fresh and fermented milk in parts of Nasarawa state reported susceptibility of *Staphylococcus aureus* to gentamicin and ciprofloxacin (100%) and chloramphenicol (77.8%). They also reported resistance to ampicillin, amoxicillin-clavulanic acid and cefoxitin (100%), tetracycline (44.4%) which closely agree with the findings from this study.

Resistance to B-lactam antibiotics observed in this study is similar to reports by Anueyiagu and Isiyaku (2015) in Jos, Plateau state, who reported 100% resistance of *Staphylococcus aureus* to B-lactam antibiotics, also Jahen *et al*. (2015) in Bangladesh reported the resistance of *Staphylococcus* species to B-lactam antibiotics. This could be attributed to the fact that B-lactam antibiotics are commonly used as dry-cow treatment process. Also, *Staphylococcus* species resistance to one B-lactam antibiotics can result in acquired resistance to other B-lactams due to similar mechanism of action (Etebu and Arikekpar 2016). Likewise, resistance to tetracycline antibiotics was significantly observed in both the *Staphylococcal* species and Enterobacteriaceae isolates from this study, this could be attributed to its use as a growth promoter and for routine prophylaxis in livestock management in Nigeria (Economou and Gousia 2015; Mouiche *et al.,* 2019). Tetracycline is also a first line antibiotic in Nigeria especially for older people with cases of gastro-intestinal infections, also in most developing countries, it is readily purchased over the counter for self-medication (Chigor *et al.,* 2010).

The detection of *tetA* and *ermB* genes in some isolates indicates the presence of multiple resistance genes, *tetA* genes are associated with tetracycline resistance, which could account for the observed resistance to tetracycline in this study, while *ermB* genes are linked to macrolide resistance, and cross resistance to antibiotics of the streptomycin and lincosamides class, which possess almost similar mechanisms of action. In spite of the observed resistance to metronidazole in this study, *nim* gene was only detected in one of the isolates, this could suggest that a different resistance mechanism is acquired. The *nim* gene is known to code for enzymes that reduce metronidazole into its inactive form, hence making the organism non-susceptible.

There was no amplification of *mecA, tetK, tetL* and *tetM* from isolates obtained from fresh raw cow milk and handlers. This could suggest that a different resistance mechanism may be in play.

Helicobacter pylori isolated from fresh raw cow milk in this study is of major concern, reports from few studies have also confirmed that foodstuff particularly milk is being assumed as a probable source of *Helicobacter pylori* transmission to human (Angelidis *et al.,* 2011; Osman *et al.,* 2015). There was no amplification of the *glmM* gene in this study, the molecular marker of the *glmM* is of different Oligonucleotide which could suggest the non-amplification observed in this study. This report agrees to the non-amplification reported by Osman *et al.* (2015). Based on cultural morphological characteristics, biochemical characteristics and the amplification of the *cagA* virulence marker of the *Helicobacter pylori* isolates from this study, it confirms relatively that *H. pylori* is readily present and can be easily transmitted to raw milk consumers via cow milk. *T*he *cagA* gene is a gene on the pathogenicity island of *Helicobacter pylori* and can be a predictive marker for this bacterium pathogenicity (Feliciano *et al.,* 2015). *Helicobacter pylori* strains can be divided into two groups: *cag* positive and *cag* negative, with *cag* positive strains being more prevalent in patients with more severe clinical symptoms (Kargar *et al.,* 2011). In most individuals, *Helicobacter pylori* infection is acquired during early childhood and can result in chronic inflammation of the gastric mucosa which could disrupt proper growth development (Mera *et al.,* 2012). Destruction of the lining of the stomach or upper part of small intestine, stomach cancer, bleeding of the stomach, severe sharp pain and weight loss are some of the damages caused by *Helicobacter pylori.*

CHAPTER SIX

6.1 CONCLUSIONS

Quality of raw milk depends on its microbial density and similarly, the production of clean milk depends largely on the hygiene of the milking personnel, the milking environment, the cleanliness of the Udder, teats, water and other containers used in the milking process.

Staphylococcal species isolated in raw milk from this study can be attributed to the fact that they are normal flora of both humans and animals, while the Enterobacteriaceae are majorly found in water and some isolated from cow teat and/or handlers, these bacteria can easily be introduced into the raw milk samples during milking practices.

Unavailability of good portable water used on the farms and/or improper udder preparations by inadequate washing and disinfecting and/or improper periodic handwashing by the handlers are reasonable means of introducing contaminations directly into the raw milk. The pre dipping solution used in cleaning the udder of the cow were performed incorrectly. Also, the towel used in cleaning were used on several cows which could increase the contamination of the herd among others.

6.2 RECOMENDATIONS

The findings in this study necessitate the need for introducing hazard analysis critical control point (HACCP) in the traditional methods of milking processes in order to reduce potential sources of microbial contaminants.

1 Prevention and control measures on the step-by-step milking procedure should be introduced appropriately. Different measures that will significantly reduce microbial contamination of raw milk in areas of;

animal health;

the sanitary state of the herd handler;

the proper pre and post dipping preparation of the cow teat and udder region and

the milking environment including the use of portable water.

2 Routine check-ups should as well be given to the herd and their handlers periodically to educate them on milking procedures.

3 Pasteurization of raw milk should be done locally before sales.

6.3 CONTRIBUTION TO KNOWLEDGE

1. Raw milk produced in Zaria has been found to be contaminated with 53.8%multiantibiotic resistant pathogens which poise serious severe health risk to its consumers and the populace.
2. *Helicobacter pylori* is pathogenic and has been found in raw cow milk produced in Zaria, Nigeria.

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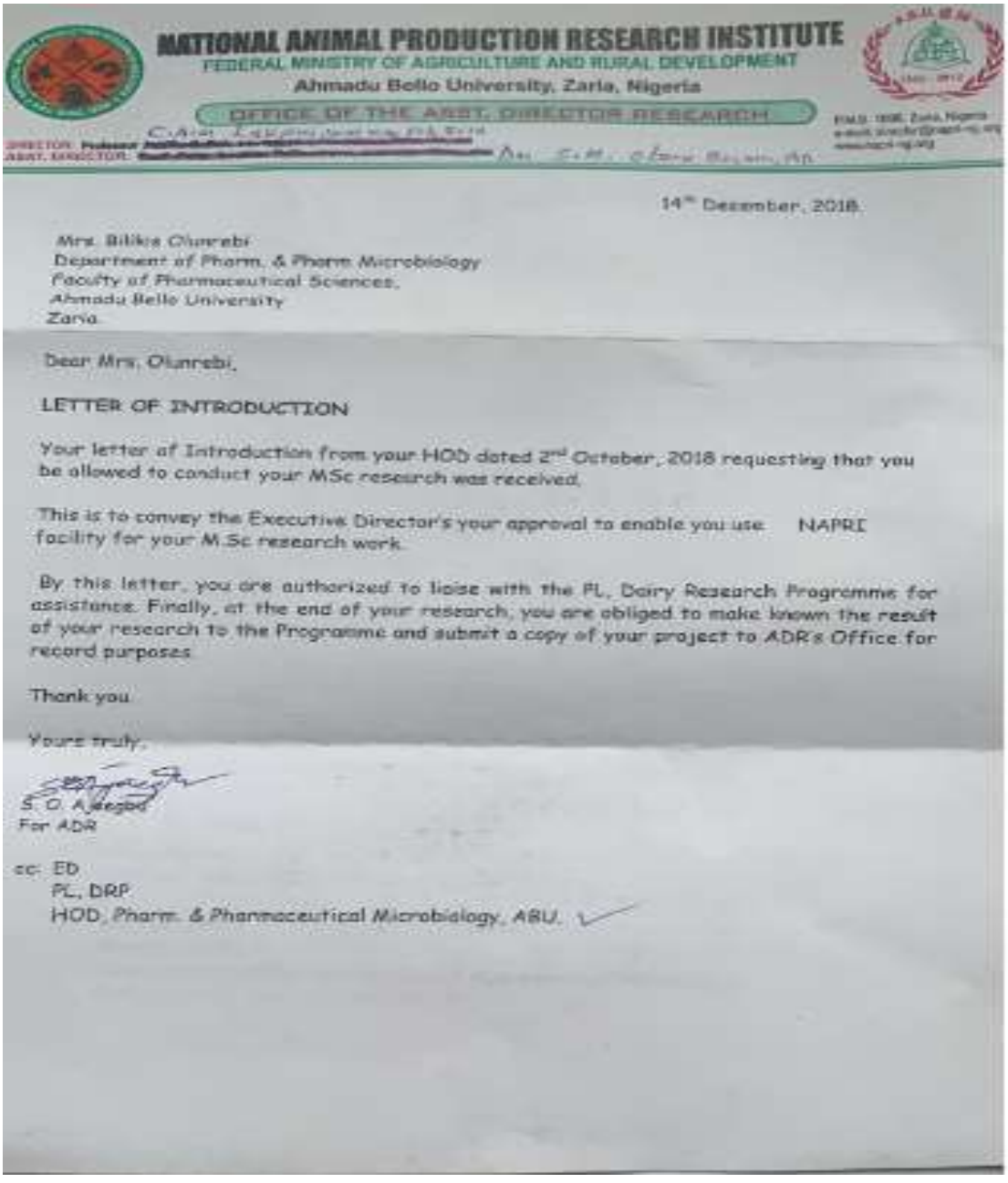
APPENDICES

Appendix I: Ethical Approval from Ahmadu Bello Ethical Committee

DIRECTORATE OF ACADEMIC PLANNING & MONITORING
AHMADU BELLO UNIVERSITY, ZARIA

APPROVAL OF RESEARCH STUDY 'MOLECULAR CHARACTERIZATION OF BACTERIA ISOLATES IN RAW MILK LOCALITY PRODUCED IN ZARIA METROPOLIS KADUNA STATE'

Appendix II: Ethical Approval from NAPRI- SHIKA Ethical Committee



NATIONAL ANIMAL PRODUCTION RESEARCH INSTITUTE
FEDERAL MINISTRY OF AGRICULTURE AND RURAL DEVELOPMENT
Ahmadu Bello University, Zaria, Nigeria
OFFICE OF THE ASST. DIRECTOR RESEARCH

14th December, 2018

Mrs. Bilikis Olunrebi
Department of Pharm. & Pharm Microbiology
Faculty of Pharmaceutical Sciences,
Ahmadu Bello University
Zaria.

Dear Mrs. Olunrebi,

LETTER OF INTRODUCTION

Your letter of Introduction from your HOD dated 2nd October, 2018 requesting that you be allowed to conduct your MSc research was received.

This is to convey the Executive Director's your approval to enable you use NAPRI facility for your M.Sc research work.

By this letter, you are authorized to liaise with the PL, Dairy Research Programme for assistance. Finally, at the end of your research, you are obliged to make known the result of your research to the Programme and submit a copy of your project to ADR's Office for record purposes

Thank you

Yours truly,

S. O. Ajeigbe
For ADR

cc: ED
PL, DRP
HOD, Pharm. & Pharmaceutical Microbiology, ABU.

Appendix III: Total Bacteria Count and Coliform Count of Fresh raw cow milk samples

S/N	Samples	TBC (Cfu/ml)	TCC (Cfu/ml)
1	ZM1	$1.8x10^4$	$1.6 x10^4$
2	ZM2	$1.4x10^4$	$0.4 x10^4$
3	ZM3	$1.0x10^4$	$0.2 x10^4$
4	ZM4	$0.3x10^4$	$0.1 x10^4$
5	SM1	$0.5x10^4$	$2.2 x10^4$
6	SM2	$3.0x10^4$	$0.4 x10^4$
7	SM3	$2.0x10^4$	$0.6 x10^4$
8	SM4	$4.2x10^4$	$0.7 x10^4$
9	KM1	$0.2x10^4$	$1.2 x10^4$
10	KM2	$0.5x10^4$	$1.1 x10^4$
11	KM3	$1.7x10^4$	$0.3 x10^4$
12	GM1	$0.2x10^4$	$0.6 x10^4$
13	GM2	$2.0x10^4$	$0.5 x10^4$
14	GM3	$1.0x10^4$	$0.6 x10^4$
15	GM4	$3.0x10^4$	$0.6 x10^4$
16	GM5	$9.8x10^4$	$2.4 x10^4$
17	GM6	$0.2x10^4$	$1.7 x10^4$
18	GM7	$0.3x10^4$	$0.1 x10^4$
19	GM8	$0.5x10^4$	$1.2 x10^4$
20	GM9	$0.4x10^4$	$0.5 x10^4$
21	GM10	$8.8x10^4$	$2.2 x10^4$
22	GM11	$5.4x10^4$	$2.8 x10^4$
23	GM12	$5.2x10^4$	$1.9 x10^4$
24	GM13	$6.1x10^4$	$2.1 x10^4$
25	GM14	$1.2x10^4$	$0.1 x10^4$
26	GM15	$0.6x10^4$	$0.1 x10^4$
27	GM16	$0.7x10^4$	$0.3 x10^4$
28	GM17	$7.5x10^4$	$3.8 x10^4$
29	GM18	$2.1x10^4$	$0.6 x10^4$
30	GM19	$0.3x10^4$	$0.1 x10^4$

Appendix III: Total Bacteria Count and Coliform Count Contd'

S/N	Samples	TBC(Cfu/ml)	TCC (Cfu/ml)
31	GM20	$0.6x10^4$	$0.2 x10^4$
32	GM21	$0.6x10^4$	$0.1 x10^4$
33	GM22	$3.6x10^4$	$1.2 x10^4$
34	GM23	$2.6x10^4$	$1.0 x10^4$
35	GM24	$6.6x10^4$	$2.4 x10^4$
36	GM25	$5.9x10^4$	$2.6 x10^4$
37	GM26	$5.4x10^4$	$3.1 x10^4$
38	GM27	$1.1x10^4$	$0.2 x10^4$
39	GM28	$2.2x10^4$	$0.3 x10^4$
40	GM29	$6.0x10^4$	$2.3 x10^4$
41	GM30	$0.7x10^4$	$0.2 x10^4$
42	GM31	$0.2x10^4$	$0.1 x10^4$

Key:

Z--- Zango farm; S---- Sabo farm; K---- Kufena farm; G---- NAPRI; M---fresh raw cow milk

Appendix IV: Methylene Blue Dye Reductase Test of Fresh raw cow milk samples

S/N	Sample	Reaction (after 8hours)
1	ZM1	no decoloration
2	ZM2	no decoloration
3	ZM3	no decoloration
4	ZM4	no decoloration
5	SM1	no decoloration
6	SM2	no decoloration
7	SM3	no decoloration
8	SM4	no decoloration
9	KM1	no decoloration
10	KM2	no decoloration
11	KM3	no decoloration
12	GM1	no decoloration
13	GM2	no decoloration
14	GM3	no decoloration
15	GM4	no decoloration
16	GM5	slight decoloration
17	GM6	no decoloration
18	GM7	no decoloration
19	GM8	no decoloration
20	GM9	no decoloration
21	GM10	slight decoloration
22	GM11	slight decoloration
23	GM12	slight decoloration
24	GM13	slight decoloration

Appendix IV: Methylene Blue Dye Reductase Test Contd'

S/N	Sample	Reaction (after 8hours)
25	GM14	no decoloration
26	GM15	no decoloration
27	GM16	no decoloration
28	GM17	slight decoloration
29	GM18	no decoloration
30	GM19	no decoloration
31	GM20	no decoloration
32	GM21	no decoloration
33	GM22	no decoloration
34	GM23	no decoloration
35	GM24	slight decoloration
36	GM25	slight decoloration
37	GM26	no decoloration
38	GM27	no decoloration
39	GM28	no decoloration
40	GM29	no decoloration
41	GM30	no decoloration
42	GM31	no decoloration

Key: Z--- Zango farm; S---- Sabo farm; K---- Kufena farm; G---- NAPRI; M---fresh raw cow milk

Appendix V: Cultural and Morphological characteristics of Bacteria Isolates from fresh raw cow milk and handlers

S/N	Samples	Morphology	
		MAC	MSA
1	ZM1	pink/red distinct	circular creamy yellow NM/F
2	ZM2	pink raised circular	mould growth (contamination)
3	ZM3	pink raised circular	yellow circular M/F
4	ZM4	pink raised circular	no growth
5	ZT1	deep pink raised	cream slightly raised
6	ZT2	deep pink raised	no growth
7	ZT3	pink raised circular	white large raised
8	ZT4	no growth	no growth
9	ZH1	deep pink raised	cream slightly raised
10	ZH2	no growth	white large raised
11	ZW	pink slightly raised	
12	SM1	no growth	creamy large circular raised
13	SM2	no growth	no growth
14	SM3	pink raised circular	golden yellow irregular
15	SM4	no growth	less circular raised
16	ST1	creamy raised circular swarming	no growth
17	ST2	deep pink raised distinct	no growth
18	ST3	creamy raised circular swarming	no growth
19	ST4	pink raised swarming	no growth
20	SH1	red pink raised circular	yellow raised circular
21	SH2	pink raised	yellow raised circular
22	SW	pink circular raised swarming	
23	KM1	red pink raised circular	no growth
24	KM2	red pink raised circular	less raised circular
25	KM3	pink swarming and light pink raised	yellow raised
26	KT1	pink raised circular	no growth

27	KT2	pink raised circular	yellow raised circular
28	KT3	pink raised circular	yellow raised circular
29	KH1	pink raised circular	large yellow raised
30	KH2	red pink raised circular	yellow circular
31	KW	pink raised circular L/F	
32	GM1	pink circular raised	yellow raised circular
33	GM2	deep pink raised circular	yellow raised circular
34	GM3	deep pink raised circular	pink circular raised
35	GM4	deep pink raised circular	no growth
36	GT1	deep pink raised circular	yellow circular M/F
37	GT2	deep pink raised circular	pink raised circular
38	GT3	deep pink raised circular	pink circular NM/F
39	GT4	deep pink raised circular	no growth
40	GH1	deep pink raised circular	yellow circular M/F
41	GH2	pink circular raised	deep yellow M/F
42	GW1	pink swarming	
43	GM5	cream swarming NL/F	yellow M/F
44	GM6	pink raised	yellow M/F
45	GM7	pink raised circular	yellow M/F
46	GM8	pink raised circular	yellow M/F
47	GM9	pink raised circular	yellow M/F
48	GM10	red pink L/F	yellow M/F
49	GM11	pink raised circular	yellow M/F
50	GM12	pink raised NL/F	yellow M/F
51	GM13	pink NL/F	yellow M/F
52	GM14	pink swarming NL/F	yellow M/F
53	GM15	pink swarming NL/F	yellow M/F
54	GM16	pink raised	yellow M/F
55	GM17	pink irregular NL/F	yellow M/F
56	GM18	pink raised	cream M/F

57	GM19	pink raised	yellow M/F
58	GM20	pink raised	yellow M/F
59	GM21	pink raised	yellow M/F
60	GM22	red pink L/F	golden yellow M/F
61	GM23	pink raised circular NL/F	yellow M/F
62	GM24	pink raised NL/F	yellow M/F
63	GM25	red pink L/F	yellow M/F
64	GM26	red pink L/F	yellow NM/F
65	GM27	red pink L/F	yellow M/F
66	GM28	pink raised L/F	yellow M/F
67	GM29	pink raised	yellow M/F
68	GM30	pink NL/F	yellow M/F
69	GM31	pink NL/F	no growth
70	GT5	red pink L/F	yellow M/F
71	GT6	red pink L/F	yellow M/F
72	GT7	red pink L/F	yellow M/F
73	GT8	pink swarming NL/F	no growth
74	GT9	pink raised circular	yellow M/F
75	GT10	pink raised circular	yellow M/F
76	GT11	pink raised circular	yellow M/F
77	GT12	pink raised circular	golden yellow M/F
78	GT13	pink cicular NLF	yellow M/F
79	GT14	pink raised circular	yellow M/F
80	GT15	pink swarming NLF	yellow M/F
81	GT16	pink raised circular	yellow M/F
82	GT17	pink raised circular	yellow M/F
83	GT18	pink raised circular	cream M/F
84	GT19	pink raised circular	yellow M/F
85	GT20	pink raised circular	yellow M/F
86	GT21	pink raised circular	yellow M/F

87	GT22	pink raised circular	yellow M/F
88	GT23	pink raised circular	yellow M/F
89	GT24	pink raised circular	no growth
90	GT25	red pink circular L/F	yellow M/F
91	GT26	pink raised circular	golden yellow M/F
92	GT27	red pink circular L/F	yellow M/F
93	GT28	no growth	no growth
94	GT29	pink raisedcircular	yellow M/F
95	GT30	pink swarming	yellow M/F
96	GT31	pink raised	golden yellow M/F
97	GH3	pink circular	yellow M/F
98	GH4	red pink circular L/F	no growth
99	GH5	pink raised circular	golden yellow M/F
100	GH6	pink circular	yellow M/F
101	GH7	pink raised circular	no growth
102	GH8	no growth	yellow M/F
103	GH9	no growth	yellow M/F
104	GH10	pink raised circular	yellow M/F
105	GW2	pink raised circular	

Key: Z--- Zango farm; S---- Sabo farm; K---- Kufena farm; G---- NAPRI; M---fresh raw cow milk; T---swab from cow teat; H---swab from handler; W---water from farm; MAC indicates MacConkey Agar; MSA indicates Mannitol Salt Agar; L/F :lactose fermenter; NL/F: non lactose fermenter; M/F: mannitol fermenter; NM/F: non mannitol fermenter

Appendix VI: Cultural Identification of Helicobacter pylori from fresh raw cow milk

S/N	Sample	Blood Agar	Gram	Shapes	Catalase	Oxidase	Hemolysis	Urease	Inference
1	KM1	NO growth							
2	KM2	Translucent	-	rods	+	+	Alpha	+	*H.pylori*
3	KM3	pale grayish	-	rods	+	+	Alpha	+	*H.pylori*
4	GM2	pale grayish	-	rods	+	+	Beta	+	*H.pylori*
5	GM3	pale grayish	-	rods	+	+	alpha	+	*H.pylori*
6	GM4	NO growth							
7	GM5	pale grayish	-	rods	+	+	alpha	+	*H.pylori*
8	GM6	NO growth							
9	GM7	NO growth							
10	GM8	NO growth							
11	GM9	Cream	-	rods	+	+	gamma	-	
12	GM10	NO growth							
13	GM11	NO growth							
14	GM12	NO growth							
15	GM13	pale grayish	-	rods	+	+	alpha	+	*H.pylori*
16	GM14	pale grayish	-	rods	+	+	alpha	+	*H.pylori*
17	GM15	pale grayish	-	rods	+	+	alpha	+	*H.pylori*
18	GM16	Cream	-	rods	+	+	gamma	-	
19	GM17	Cream	-	rods	+	+	gamma	-	
20	GM18	pale grayish	-	rods	+	+	alpha	+	*H.pylori*
21	GM19	NO growth							
22	GM20	pale grayish	-	rods	+	+	gamma	-	
23	GM21	NO growth							
24	GM22	NO growth							
25	GM23	pale grayish	-	rods	+	+	alpha	+	*H.pylori*
26	GM24	Cream	-	rods	+	+	gamma	-	
27	GM25	pale grayish	-	rods	+	+	alpha	+	*H.pylori*
28	GM26	NO growth							
29	GM27	pale grayish	-	rods	+	+	alpha	+	*H.pylori*
30	GM28	NO growth							
31	GM29	NO growth							
32	GM30	NO growth							
33	GM31	NO growth							

Key:KM1, KM2 e.t.c indicates samples of raw milk collected from Kufena farm
GM2, GM3 e.t.c indicates samples of raw milk collected from NAPRI
NB: + indicates positive reaction; - indicates negative reaction

Appendix VII: Biochemical Identification of Staphylococci specie

S/N	Sample	Morphology		Gram	Catalase	Coagulase	Bacteria isolated
		NA	MSA				
1	ZM1	white	yellow	+	+	-	*Staphylococcus hyicus*
2	ZM3	gold	yellow	+	+	+	*Staphylococcus aureus*
3	ZT1	gold	cream	+	+	-	*Staphylococcus chromegenes*
4	ZT3	yellow	cream	+	+	-	*Staphylococcus xylosus*
5	SM1	white	cream	+	+	-	*Staphylococcus hyicus*
6	SM3	white	gold	+	+	-	*Staphylococcus chromegenes*
7	KM2	white	cream	+	+	+	*Staphylococcus aureus*
8	KT3	gold	yellow	+	+	+	*Staphylococcus aureus*
9	KH1	white	yellow	+	+	-	*Staphylococcus intermedius*
10	GM1	white	yellow	+	+	+	*Staphylococcus aureus*
11	GM2	gold	yellow	+	+	+	*Staphylococcus aureus*
12	GM5	cream	yellow	+	+	-	*Micrococcus luteus*
13	GM10	white	yellow	+	+	+	*Staphylococcus aureus*
14	GM13	white	yellow	+	+	-	*Staphylococcus hyicus*
15	GM14	white	yellow	+	+	+	*Staphylococcus aureus*
16	GM17	cream	yellow	+	+	+	*Staphylococcus aureus*
17	GM22	white	yellow	+	+	+	*Staphylococcus aureus*
18	GM24	cream	yellow	+	+	+	*Staphylococcus aureus*
19	GM26	cream	cream	+	+	-	*Micrococcus luteus*
20	GM28	white	yellow	+	+	-	*Staphylococcus hyicus*

Key: Z--- Zango farm; S---- Sabo farm; K---- Kufena farm; G---- NAPRI M---fresh raw cow milk; T---swab from cow teat; H---swab from handler; W---water from farm

+ indicates positive reaction; - indicates negative reaction

Appendix VIII: Biochemical Identification of Enterobacteriaceae

S/N	Sample	Morphology		Gram	Catalase	Oxidase	Bacteria isolated
		NA	MAC				
1	GH4	yellow	red pink circular	-	+	-	*S.choleraesus*
2	GM23	yellow	pink raised cicular	-	+	-	*Acinetobacter iwoffi*
3	GM7	cream	pink raised cicular	-	+	-	*Acinetobacter iwoffi*
4	GM9	cream	pink raised cicular	-	+	-	*Acinetobacter iwoffi*
5	GT10	cream	pink raised cicular	-	+	-	*Acinetobacter iwoffi*
6	KT4	white	pink raised cicular	-	+	-	*Acinetobacter iwoffi*
7	GT3	cream	deep pink raised circular	-	+	-	*Acinetobacter iwoffi*
8	GM4	white	deep pink raised circular	-	+	-	*Pantoea agglomerans*
9	GT4	white	deep pink raised circular	-	+	-	*Providencia stuartii*
10	GM3	white	deep pink raised circular	-	+	-	*Hafnia alveri*
11	GM2	white	deep pink raised circular	-	+	-	*Pantoea agglomerans*
12	GT1	white	deep pink raised circular	-	+	-	*Acinetobacter iwoffi*
13	ZT3	yellow	pink raised cicular	-	+	-	*Providencia stuartii*
14	KM2	white	red pink circular	-	+	-	*Citrobacter freundi*
15	KH1	cream	pink raised cicular	-	+	-	*Proteus vulgaris*
16	GH2	cream	pink raised cicular	-	+	-	*Proteus mirabilis*
17	GH1	yellow	deep pink raised circular	-	+	-	*Proteus vulgaris*
18	KT1	yellow	pink raised cicular	-	+	-	*Proteus vulgaris*
19	GT2	yellow	deep pink raised circular	-	+	-	*Proteus vulgaris*
20	KMI	cream	red pink circular	-	+	-	*Proteus vulgaris*
21	KT3	cream	pink raised cicular	-	+	-	*Proteus vulgaris*
22	KH2	cream	red pink circular	-	+	-	*Yersinia enterocolitica*
23	KM3	cream	pink swarming	-	+	-	*Proteus vulgaris*
24	ZT1	white	deep pink raised circular	-	+	-	*Acinetobacter iwoffi*
25	SH1	white	red pink raised circular	-	+	-	*Escherichia coli*
26	ZW	cream	pink sightly raised	-	+	-	*Acinetobacter iwoffi*
27	ZH1	white	deep pink raised circular	-	+	-	*Klebsiella pneumonia*

28	ST2	cream	deep pink raised circular	-	+	-	*Klebsiella oxytoca*
29	GM6	cream	pink raised cicular	-	+	-	*Acinetobacter iwoffi*
30	GM11	cream	pink raised cicular	-	+	-	*Acinetobacter iwoffi*
31	GM20	yellow	pink raised circular	-	+	-	*Acinetobacter iwoffi*
32	GM28	yellow	pink raised L/F	-	+	-	*Escherichia coli*
33	GM19	white	pink raised	-	+	-	*Acinetobacter iwoffi*
34	GH6	white	pink circular	-	+	-	*Pantoea agglomerans*
35	GM31	cream	pink NL/F	-	+	-	*Acinetobacter iwoffi*
36	GH3	yellow	pink circular	-	+	-	*Acinetobacter iwoffi*
37	KW	white	pink raised circular L/F	-	+	-	*Escherichia coli*
38	SW	cream	pink raised swarming	-	+	-	*Citrobacter youngae*
39	GT31	yellow	pink raised	-	+	-	*Hafnia alveri*
40	GM27	cream	red pink circular	-	+	+	*Aeromonas hydrophila*
41	GH10	yellow	pink raised circular	-	+	+	*Moraxella specie*
42	GM24	cream	pink raised NL/F	-	+	+	*Pseudomonas aeruginosa*
43	GM13	cream	pink NL/F	-	+	+	*Burkholderia pseudomallei*
44	GM9	cream	pink raised circular	-	+	+	*Moraxella specie*
45	GW1	cream	pink swarming	-	+	+	*Moraxella specie*
46	ZM3	white	pink raised circular	-	+	+	*Pasteurella multocida*
47	KM3	white	pink raised circular	-	+	+	*Aeromonas caviae*
48	GM5	yellow	cream swarming NL/F	-	+	+	*Plesiomonas shigelloides*
49	GT16	white	pink raised	-	+	+	*Moraxella specie*
50	GM14	cream	pink raised circular NL/F	-	+	+	*Aeromonas hydrophila*
51	GM15	cream	pink swarming NL/F	-	+	+	*Pseudomonas aeruginosa*
52	SM3	cream	pink swarming	-	+	+	*Pseudomonas aeruginosa*
53	ST3	cream	cream swarming NL/F	-	+	+	*Flavobacterium odoratum*
54	GM25	cream	red pink circular	-	+	+	*Moraxella specie*
55	GM16	cream	pink raised	-	+	+	*Moraxella specie*
56	SH2	cream	pink raised circular	-	+	+	*Pseudomonas aeruginosa*
57	GM17	cream	pink irregular NL/F	-	+	+	*Moraxella specie*

58	GM18	cream	pink raised	-	+	+	*Vibrio parahaemolyticus*
59	GT12	cream	pink circular	-	+	+	*Weeksela zoohelcum*
60	GM30	white	pink NL/F	-	+	+	*Aeromonas hydrophila*

Key:

Z--- Zango farm; S---- Sabo farm; K---- Kufena farm; G---- NAPRI

M---fresh raw cow milk; T---swab from cow teat; H---swab from handler; W---water from farm

+ indicates positive reaction; - indicates negative reaction L/F :lactose fermenter; NL/F: non lactose fermenter

Appendix IX: Antimicrobial susceptibility on *Staphylococcal species* and *Micrococcus leutus*

S/N	Sample	Gram	Bacteria isolates	FOX	TET	CIP	AMP	CN	ERY	AMC	CHL	MAR(%)
1	GM2	+	*Staphylococcus aureus*	R	S	S	R	S	S	R	S	0.4
2	ZT3	+	*Staphylococcus xylosus*	R	R	R	R	S	S	S	S	0.5
3	GM13	+	*Staphylococcus hyicus*	R	R	S	R	R	S	R	S	0.6
4	GM14	+	*Staphylococcus aureus*	S	R	S	R	S	S	R	S	0.4
5	GM1	+	*Staphylococcus aureus*	R	S	S	R	S	S	R	S	0.4
6	ZT1	+	*Staphylococcus chromogens*	R	R	R	R	R	S	R	S	0.8
7	SM1	+	*Staphylococcus hyicus*	R	R	S	R	S	R	R	S	0.6
8	GM10	+	*Staphylococcus aureus*	R	R	S	S	S	S	R	S	0.4
9	KT3	+	*Staphylococcus aureus*	S	R	S	S	R	S	R	-	0.4
10	KM2	+	*Staphylococcus aureus*	R	R	S	S	S	S	R	S	0.4
11	GM17	+	*Staphylococcus aureus*	R	R	S	R	S	R	R	S	0.6
12	KH1	+	*Staphylococcus intermedius*	R	R	S	S	R	S	R	S	0.5
13	SM3	+	*Staphylococcus chromogens*	R	S	R	R	S	R	R	S	0.6
14	ZM1	+	*Staphylococcus hyicus*	R	S	S	S	S	S	S	S	0.1
15	GM5	+	*Micrococcus leutus*	R	R	S	R	S	R	R	S	0.6
16	GM24	+	*Staphylococcus aureus*	R	R	S	R	S	R	R	R	0.8
17	GM26	+	*Micrococcus leutus*	S	R	S	S	S	S	S	S	0.1
18	ZM3	+	*Staphylococcus aureus*	R	S	S	S	S	S	R	S	0.3
19	GM22	+	*Staphylococcus aureus*	R	R	S	R	S	S	R	S	0.5
20	GM28	+	*Staphylococcus hyicus*	R	R	S	R	S	S	R	S	0.5

Key: Z--- Zango farm; S---- Sabo farm; K---- Kufena farm; G---- NAPRI M---fresh raw cow milk; T---swab from cow teat; H---swab from handler; W--- water from farm

+ indicates positive reaction; R indicates resistance to antimicrobial ; S indicates susceptibility to antimicrobial; FOX= Cefoxitin ; ERY= Erythromycin; AMP= Ampicillin; TET= Tetracycline; AMC= Amoxi-clavulanic acid ; CN= Gentamicin; CIP= Ciprofloxacin; CHL= Chloramphenicol

Appendix X: Antimicrobial Susceptibility on Enterobacteriaceae species

S/N	Sample	Gram	Bacteria isolates	CAZ	TET	CIP	AMP	CN	ERY	AMC	CHL	MTZ	MARI(%)
1	GM4	-	*Pantoea agglomerance*	S	R	S	S	S	R	R	S	-	0.38
2	KM2	-	*Citrobacter freundi*	R	S	S	S	S	R	S	S	R	0.33
3	GM7	-	*Acinetobacter iwoffii*	R	S	S	S	S	S	R	S	R	0.33
4	SW	-	*Citrobacter youngae*	R	R	R	R	R	R	R	S	R	0.89
5	KH1	-	*Proteus vulgaris*	R	S	S	S	S	R	S	S	R	0.33
6	GH10	-	*Moraxella specie*	R	R	S	S	S	S	S	S	R	0.33
7	GM15	-	*Pseudomonas aeruginosa*	R	R	S	R	S	R	R	R	R	0.78
8	GM20	-	*Acinetobacter iwoffii*	R	R	S	R	R	R	R	R	R	0.89
9	GT16	-	*Moraxella specie*	R	R	S	S	S	S	S	S	R	0.33
10	GH3	-	*Acinetobacter iwoffii*	R	S	S	S	S	S	R	S	R	0.33
11	GM16	-	*Moraxella specie*	R	R	S	R	S	S	R	S	R	0.56
12	GM17	-	*Moraxella specie*	R	R	S	R	R	R	R	R	R	0.89
13	GM14	-	*Aeromonas hydrophila*	R	R	S	R	S	R	R	R	R	0.78
14	ZM3	-	*Pasteurella multocida*	R	R	S	R	S	R	R	R	R	0.78
15	GW	-	*Moraxella specie*	R	R	S	S	S	S	S	S	R	0.33
16	SH2	-	*Pseudomonas aeruginosa*	R	R	S	R	S	R	R	R	R	0.78
17	GM11	-	*Acinetobacter iwoffii*	R	S	S	S	S	R	R	S	R	0.44
18	GM23	-	*Acinetobacter iwoffii*	R	R	S	R	S	R	R	R	R	0.78
19	KM3	-	*Proteus vulgaris*	R	R	R	R	S	R	R	S	R	0.78
20	KT1	-	*Proteus vulgaris*	R	R	R	S	S	R	S	S	R	0.56
21	ST2	-	*Klebsiella oxytoca*	R	R	S	R	S	R	R	S	R	0.67
22	SH1	-	*Escherichia coli*	R	S	S	S	S	S	S	S	R	0.22
23	GT10	-	*Acinetobacter iwoffii*	R	S	S	S	S	S	R	S	R	0.33
24	KH2	-	*Yersinia enterolitica*	R	R	R	R	S	R	R	S	R	0.78
25	KT4	-	*Acinetobacter iwoffii*	S	R	R	R	S	R	R	S	R	0.67
26	GM19	-	*Acinetobacter iwoffii*	R	R	S	R	S	S	R	S	R	0.56

27	GH6	-	*Pantoea agglomerance*	R	S	S	S	S	S	S	S	R	0.22
28	ZT1	-	*Acinetobacter iwoffii*	R	S	S	S	S	S	S	S	R	0.22
29	GT12	-	*Weeksela zoohelcum*	R	S	S	S	S	S	S	S	R	0.22
30	GM31	-	*Acinetobacter iwoffii*	R	-	-	R	S	-	S	-	R	0.6
31	GH4	-	*S.choleraesus*	R	R	S	R	S	-	R	R	R	0.75
32	GM9	-	*Acinetobacter iwoffii*	R	-	-	S	S	-	R	-	R	0.6
33	KM1	-	*Proteus vulgaris*	R	-	-	R	S	-	R	-	R	0.8
34	GM28	-	*Escherichia coli*	R	S	S	R	S	-	R	S	R	0.5
35	GT4	-	*Providencia stuarti*	R	-	-	S	S	-	S	-	R	0.4
36	GM6	-	*Acinetobacter iwoffii*	R	S	S	R	S	-	R	-	R	0.57
37	KT3	-	*Proteus vulgaris*	R	R	S	-	S	R	S	S	R	0.5
38	GM2	-	*Pantoea agglomerance*	S	R	S	-	S	R	-	S	R	0.43
39	GM3	-	*Hafnia alveri*	S	R	R	-	S	R	R	S	R	0.63
40	GT1	-	*Acinetobacter iwoffii*	R	R	S	-	S	R	S	S	R	0.5
41	ZH1	-	*Klebsiella pneumonia*	R	S	S	R	S	R	R	S	R	0.56
42	ZT3	-	*Providencia stuarti*	R	R	R	R	S	R	S	S	R	0.67
43	ZW	-	*Acinetobacter iwoffii*	S	S	S	-	S	R	R	S	R	0.38
44	GH1	-	*Proteus vulgaris*	R	R	R	R	S	R	S	S	R	0.67
45	GH2	-	*Proteus mirabilis*	R	R	S	R	S	R	S	S	R	0.56
46	GT2	-	*Proteus vulgaris*	R	R	R	-	S	R	R	S	R	0.75
47	GM18	-	*Vibrio parahaemolyticus*	R	R	S	R	S	S	R	S	R	0.56
48	GM13	-	*Burkholderia pseudomallei*	S	R	R	-	S	R	R	S	R	0.63
49	SM3	-	*Pseudomonas aeruginosa*	R	R	R	-	S	R	R	S	R	0.75
50	GM30	-	*Aeromonas hydrophila*	R	S	S	-	S	R	R	S	R	0.5
51	GM25	-	*Moraxella specie*	R	R	S	R	S	R	R	S	R	0.67
52	GM27	-	*Aeromonas hydrophila*	R	R	S	R	S	R	R	S	R	0.67
53	GM5	-	*Plesiomonas shigelloides*	R	S	S	R	S	R	R	R	R	0.67
54	GT31	-	*Hafnia alveri*	No Growth Along Streaked Lines And No Zone Of Inhibition (Repeated *3)									

CAZ =Ceftazidime AMP=Ampicillin AMC Amoxi-Clavulanic acid TET Tetracycline CN= Gentamicin CHL= Chloramphenicol CIP= Ciprofloxacin ERY=Erythromycin MTZ= Metronidazole

1% agarose gel combination: 1.0g agarose in 250mls of 1x concentration of Tris borate ethylene diamine tetra acetate (TBE), 98mls distilled water

ROUGH WORK AND RAW DATA

TABLE

	ALL	MILK	HANDLER	TEAT	WATER	
NEGATIVE	03	01S	-	01G+01Z	-	2.9
POSITIVE						97.1
POLYMICROBIAL	76	29G+02K+01S+02Z 34	06G+02K+02S+01Z 11	27G+02K+02Z 31	-	
SINGLE MICROBE	26	02G+01K+02S+02Z 07	04G+01Z+ 05	03G+01K+04S+01Z 09	02G+01K+01S+01Z 05	
	105					100

NO GROWTH	GRAM NEGATIVE	GRAM POSITIVE	GRAM POSITIVE AND NEGATIVE
03	21	05	76

		GRAM NEGATIVE	GRAM POSITIVE	TYPE	
GT28		no growth	no growth		
SM2		no growth	no growth		
ZT4		no growth	no growth		
GH1		+	+	MIXED	
GH10		+	+	MIXED	
GH2		+	+	MIXED	
GH3		+	+	MIXED	
GH5		+	+	MIXED	
GH6		+	+	MIXED	
GM1		+	+	MIXED	
GM10		+	+	MIXED	
GM11		+	+	MIXED	
GM12		+	+	MIXED	
GM13		+	+	MIXED	
GM14		+	+	MIXED	
GM15		+	+	MIXED	
GM16		+	+	MIXED	
GM17		+	+	MIXED	
GM18		+	+	MIXED	
GM19		+	+	MIXED	
GM2		+	+	MIXED	
GM20		+	+	MIXED	
GM21		+	+	MIXED	
GM22		+	+	MIXED	

GM23		+	+	MIXED	
GM24		+	+	MIXED	
GM25		+	+	MIXED	
GM26		+	+	MIXED	
GM27		+	+	MIXED	
GM28		+	+	MIXED	
GM29		+	+	MIXED	
GM3		+	+	MIXED	
GM30		+	+	MIXED	
GM5		+	+	MIXED	
GM6		+	+	MIXED	
GM7		+	+	MIXED	
GM8		+	+	MIXED	
GM9		+	+	MIXED	
GT1		+	+	MIXED	
GT10		+	+	MIXED	
GT11		+	+	MIXED	
GT12		+	+	MIXED	
GT13		+	+	MIXED	
GT14		+	+	MIXED	
GT15		+	+	MIXED	
GT16		+	+	MIXED	
GT17		+	+	MIXED	
GT18		+	+	MIXED	
GT19		+	+	MIXED	
GT2		+	+	MIXED	
GT20		+	+	MIXED	
GT21		+	+	MIXED	
GT22		+	+	MIXED	
GT23		+	+	MIXED	
GT25		+	+	MIXED	
GT26		+	+	MIXED	
GT27		+	+	MIXED	
GT29		+	+	MIXED	
GT3		+	+	MIXED	
GT30		+	+	MIXED	
GT31		+	+	MIXED	
GT5		+	+	MIXED	
GT6		+	+	MIXED	
GT7		+	+	MIXED	
GT9		+	+	MIXED	
KH1		+	+	MIXED	
KH2		+	+	MIXED	
KM2		+	+	MIXED	
KM3		+	+	MIXED	
KT2		+	+	MIXED	
KT3		+	+	MIXED	

SH1		+	+	MIXED	
SH2		+	+	MIXED	
SM3		+	+	MIXED	
ZH1		+	+	MIXED	
ZM1		+	+	MIXED	
ZM3		+	+	MIXED	
ZT1		+	+	MIXED	
ZT3		+	+	MIXED	
GH4		+	no growth	SINGLE	
GH7		+	no growth	SINGLE	
GH8		no growth	+	SINGLE	
GH9		no growth	+	SINGLE	
GM31		+	no growth	SINGLE	
GM4		+	no growth	SINGLE	
GT24		+	no growth	SINGLE	
GT4		+	no growth	SINGLE	
GT8		+	no growth	SINGLE	
GW1		+		SINGLE	
GW2		+		SINGLE	
KM1		+	no growth	SINGLE	
KT1		+	no growth	SINGLE	
KW		+		SINGLE	
SM1		no growth	+	SINGLE	
SM4		no growth	+	SINGLE	
ST1		+	no growth	SINGLE	
ST2		+	no growth	SINGLE	
ST3		+	no growth	SINGLE	
ST4		+	no growth	SINGLE	
SW		+		SINGLE	
ZH2		no growth	+	SINGLE	
ZM2		+	mould growth (contamination)	SINGLE	
ZM4		+	no growth	SINGLE	
ZT2		+	no growth	SINGLE	
ZW		+		SINGLE	

S/N	Bacteria isolates	(%)				
		PDR	XDR	MDR	nMDR	
	MILK	0	G8+K1+S1+Z1	G14+K2		
	TEAT	0	G1+K1+S1+Z1	G3+K2	G3+Z1	
	HANDLER	0	G2+K1+S1	G3+K1+Z1	G1+S1	
	WATER	0	S1	G1+Z1		

S/N	Bacteria isolates	(%)			
		PDR	XDR	MDR	nMDR
	MILK	0	G1	G9+K1+S2	G1+Z2

	TEAT	0	Z1	K1+Z1	
	HANDLER	0		K1	
	WATER	0			

S/N	Bacteria isolates	(%)				
		PDR	XDR	MDR	nMDR	
	MILK	0	11	16		27
	TEAT	0	4	5	4	13
	HANDLER	0	4	5	2	11
	WATER	0	1	2		3
			20	28	6	54

S/N	Bacteria isolates	(%)			
		PDR	XDR	MDR	nMDR
	MILK	0	G1	G9+K1+S2	G1+Z2
	TEAT	0	Z1	K1+Z1	
	HANDLER	0		K1	
	WATER	0			

S/N	Bacteria isolates	(%)				
		PDR	XDR	MDR	nMDR	
	MILK	0	01	12	03	16
	TEAT	0	01	02		03
	HANDLER	0		01		01
	WATER	0				
			02	15	03	20

S/N	Bacteria isolates	(%)						
		PDR	XDR	MDR	nMDR			
	MILK	0	11+1 12	16 +12 28	03	27	16	43
	TEAT	0	4+1 5	5+1 6	4+2 6	13	03	16
	HANDLER	0	4	5+1 6	2	11	01	12
	WATER	0	1	2		3	00	03
			20	28	6	54	20	74

S/N	Bacteria isolates	(%)						
		PDR	XDR	MDR	nMDR			
	MILK	0	11	16		27	16	43
	TEAT	0	4	5	4	13	03	16

	HANDLER	0	4	5	2	11	01	12
	WATER	0	1	2		3	00	03
			20	28	6	54	20	74

SAMPLE SOURCE * SAMPLING LOCATION Crosstabulation						
			SAMPLING LOCATION			
			GIWA	KUFENA	SABO	ZANGO
SAMPLE SOURCE	MILK	Count	35	5	3	3
		Expected Count	28.8	7.5	4.6	5.2
		Std. Residual	1.2	-.9	-.7	-1.0
		Adjusted Residual	2.9	-1.5	-1.2	-1.6
	TEAT	Count	8	4	2	4
		Expected Count	11.3	2.9	1.8	2.0
		Std. Residual	-1.0	.6	.1	1.4
		Adjusted Residual	-1.8	.8	.2	1.7
	HANDLER	Count	6	3	2	1
		Expected Count	7.5	2.0	1.2	1.4
		Std. Residual	-.5	.8	.7	-.3
		Adjusted Residual	-1.0	.9	.8	-.3
	WATER	Count	1	1	1	1
		Expected Count	2.5	.7	.4	.5
		Std. Residual	-.9	.4	.9	.8
		Adjusted Residual	-1.6	.5	1.0	.9
Total		Count	50	13	8	9
		Expected Count	50.0	13.0	8.0	9.0

SAMPLE SOURCE * SAMPLING LOCATION Crosstabulation			
			Total
SAMPLE SOURCE	MILK	Count	46
		Expected Count	46.0
		Std. Residual	
		Adjusted Residual	
	TEAT	Count	18
		Expected Count	18.0
		Std. Residual	
		Adjusted Residual	
	HANDLER	Count	12
		Expected Count	12.0
		Std. Residual	
		Adjusted Residual	
	WATER	Count	4

		Expected Count	4.0
		Std. Residual	
		Adjusted Residual	
Total		Count	80
		Expected Count	80.0

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	11.081[a]	9	.270
Likelihood Ratio	10.637	9	.301
Linear-by-Linear Association	5.665	1	.017
N of Valid Cases	80		

RESISTANCE CLASSIFICATION					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	XDR	22	27.5	29.7	29.7
	MDR	43	53.8	58.1	87.8
	nMDR	9	11.3	12.2	100.0
	Total	74	92.5	100.0	
Missing	System	6	7.5		
Total		80	100.0		

SAMPLE SOURCE * RESISTANCE CLASSIFICATION Crosstabulation						
			RESISTANCE CLASSIFICATION			Total
			XDR	MDR	nMDR	
SAMPLE SOURCE	MILK	Count	12	28	3	43
		Expected Count	12.8	25.0	5.2	43.0
		Std. Residual	-.2	.6	-1.0	
		Adjusted Residual	-.4	1.4	-1.6	
	TEAT	Count	5	7	4	16
		Expected Count	4.8	9.3	1.9	16.0
		Std. Residual	.1	-.8	1.5	
		Adjusted Residual	.2	-1.3	1.8	
	HANDLER	Count	4	6	2	12
		Expected Count	3.6	7.0	1.5	12.0
		Std. Residual	.2	-.4	.4	
		Adjusted Residual	.3	-.6	.5	
	WATER	Count	1	2	0	3

		Expected Count	.9	1.7	.4	3.0
		Std. Residual	.1	.2	-.6	
		Adjusted Residual	.1	.3	-.7	
Total		Count	22	43	9	74
		Expected Count	22.0	43.0	9.0	74.0

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	4.915[a]	6	.555
Likelihood Ratio	4.964	6	.548
Linear-by-Linear Association	.016	1	.899
N of Valid Cases	74		

SAMPLING LOCATION * RESISTANCE CLASSIFICATION Crosstabulation						
			RESISTANCE CLASSIFICATION			Total
			XDR	MDR	nMDR	
SAMPLING LOCATION	GIWA	Count	12	30	5	47
		Expected Count	14.0	27.3	5.7	47.0
		Std. Residual	-.5	.5	-.3	
		Adjusted Residual	-1.0	1.3	-.5	
	KUFENA	Count	3	8	0	11
		Expected Count	3.3	6.4	1.3	11.0
		Std. Residual	-.1	.6	-1.2	
		Adjusted Residual	-.2	1.1	-1.3	
	SABO	Count	4	2	1	7
		Expected Count	2.1	4.1	.9	7.0
		Std. Residual	1.3	-1.0	.2	
		Adjusted Residual	1.7	-1.7	.2	
	ZANGO	Count	3	3	3	9
		Expected Count	2.7	5.2	1.1	9.0
		Std. Residual	.2	-1.0	1.8	
		Adjusted Residual	.3	-1.6	2.1	
Tostal		Count	22	43	9	74
		Expected Count	22.0	43.0	9.0	74.0

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	9.551[a]	6	.145
Likelihood Ratio	9.827	6	.132
Linear-by-Linear Association	.001	1	.981
N of Valid Cases	74		