DIVERSITY OF YEASTS AND COLIFORMS ASSOCIATED WITH BOVINE SUBCLINICAL MASTITIS IN PERIURBAN DAIRY FARMS IN KADUNA METROPOLIS, KADUNA STATE, NIGERIA.

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY IN VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICNE

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE AHMADU BELLO UNIVERSITY ZARIA, NIGERIA

FEBRUARY, 2017

DECLARATION

I declare that the work in thisThesis entitled **"Diversity of yeasts and coliforms associated with bovine subclinical mastitis in periurban dairy farms in Kaduna metropolis, Kaduna State, Nigeria"**was carried out by me in the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Elsie Uduak MBUK_____ _____

Name of student Signature Date

CERTIFICATION

This thesis entitled **"DIVERSITY OF YEASTS AND COLIFORMS ASSOCIATED WITH BOVINE SUBCLINICAL MASTITIS IN PERIURBAN DAIRY FARMS IN KADUNA METROPOLIS, KADUNA STATE, NIGERIA"** by Elsie Uduak MBUK meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria,and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This Thesis is dedicated to my lovely husband Polite and daughters Otito Ruby and Osahambili Alma Onwuhafua.

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ABSTRACT

This study aimed to examine the diversity of yeasts and coliforms associated with bovine subclinical mastitis in periurban dairy farms in Kaduna Metropolis, Kaduna State, Nigeria, including the use of molecular techniques. A cross-sectional study was conducted on twenty-six dairy farms drawn from four local government areas in the state. A total of 300 composite milk samples were collected from 300 cows-in-milking and examined. The result show that: 37 (12.3%) fungal isolates were identified using the API 20C AUX as; (9) *Candida albicans*, (4) *Candida famata*, (4) *Candida krusei*, (1) *Candida boidinii*, (1) *Candida pelliculosa*,(1) *Candida lusitaniae*,(5) *Trichosporon mucoides*, (1)*Cryptococcus humicola*, (1) *Cryptococcus laurentii*, (1) *Cryptococcus albidus*, (2) *Saccharomyces cerevisiae*, (2) *Stephanoascus ciferrii*, (2) *Rhodotorula mucilaginosa*, (1) *Kloeckera spp*., (1) *Kodamaea ohmeri*and (1) *Geotrichum capitatum*.All *C. albicans* had the ability to survive high temperature, produced germ tubes in human serum and formed chlamydospores in corn meal-tween 80 Agar. *In-vitro* sensitivity test using five antimycotic agents showedthat 85% of the isolates were sensitive to amphotericin B, followed by griseofulvin, nystatin, variconazole and fluconazole in decreasing order. Nested PCR of the D1 / D2 domains of the 26S rRNA gene of 11 yeast isolates showed they all had a distinct band- 600bp. DNA sequencing and GenBank BLASTN of the eleven genes identified them as: (3) *Candida albicans*,(1) *Saccharomyces cerevisiae* and (7) *Pichia Kudriavzevii*(*Candida krusei*). The D1/D2 26S rRNA gene sequences were 93-100% identical for yeast isolates within the same species. Phylogenetic reconstruction based on the D1/D2 26S rRNA gene sequences grouped them into 3 clusters and showed heterogeneity in *C. albicans*. A 10.3% prevalence was recorded for coliform organisms using the Microgen GN-ID A+B Kit (Medica-Tec^{TM}), these were; (1) *Enterobacter cloacae*, (1) *Enterobacter aerogenes*, (2)*Enterobacter gergoviae*, (2)*Citrobacterfreundii*, (1) *Citrobacter koseri*, (11) *Klebsiellapneumoniae*, (5) *Klebsiella*

oxytoca, (4)*Serratia marcescens*, (3) *Proteus mirabilis* and (1) *Pantoea agglomerans.In-vitro* sensitivity test using seven commonly available antibiotics showed that all the isolates were sensitive to amoxicillin followedby ciprofloxacin, gentamicin, chloramphenicol, streptomycin, tetracycline and erythromycin in decreasing order. Eighty-nine (29.7%) cow milk samples were CMT positive of these: 13(4.3%) were contaminated with yeasts only, 7(2.3%) contaminated with only coliforms while, 24(8%) were contaminated with both yeasts and coliforms. Age, parity number, stage of lactation, management system, milking hygiene and presence of lesion on udder/teat were found to be significantly associated ($p<0.05$) with the prevalence of mastitis in cows. The lowest prevalence (24%; 48 of 200) was recorded in cows within 3–4 years of age while, the highest (60.6%; 20 of 33) was in cows aged above 5 years. Stage of lactation was significant with the prevalence of mastitis being the highest (45.5%; 30 of 66) during the initial stage of lactation (0 to 5 month). It was concluded that, the relatively high prevalence of yeasts and coliforms in bovine subclinical mastitis in dairy herds could significantly reduce milk production and cause economic losses. The milk samples contained diverse yeast species including isolates of the pathogenic yeast *C. albicans* and this raises the possibility of milk and dairy products being vehicles for transmission of pathogenic yeasts. It is therefore recommended that the farmers practice good milking hygiene, milk clinically infected cows last, cull chronic mastitis cases, treat clinically infected cows and administer dry period therapy to their cows. As this will go a long way to reduce the prevalence of yeasts and coliformsin subclinical mastitis in periurban dairy farms in Kaduna metropolis, Kaduna State, Nigeria.

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LIST OF ABBREVIATONS

AIDS	Acquired immunodeficiency syndrome
CMT	California Mastitis Test
CLSI	Clinical and Laboratory Standards Institute
CFU	Colony Forming Unit
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
LPS	Lipopolysaccharide
LGA	Local Government Area
MRVP	Methyl Red VogeS Proskauer
NCBI	National Center for Biotechnology Information
NMC	National Mastitis Council
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
SDA	Sabouraud Dextrose Agar
SCC	Somatic Cell Count
SPSS	Statistical Package for Social Science
SIM	Sulfide, Indole, Motility
TSI	Triple Sugar Iron agar
USD	United States Dollar
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Bovine mastitis is the inflammatory response of the udder to noxious agents that can be either infectious or non-infectious characterized by pathological alterations in mammary tissues, compositional changes in milk, elevated milk somatic cells, and pain to the affected animal resulting in reductions of milk yield and quality (Matofari *et al*., 2005; Radostits *et al*., 2007). Most frequently the aetiology are infectious and organisms as diverse as bacteria, mycoplasmas, yeasts and algae have been implicated (Smith and Hogan, 2001; Krukowski *et al*., 2006; Kivaria and Noordhuizen, 2007).

Bovine mycotic mastitis is usually caused by yeasts, but mastitis due to filamentous fungi mostly *Aspergillus fumigatus* has been reported (LasHeras *et al*., 2000). Mycotic mastitis occurs as sporadic cases affecting a small percentage of cows, or as outbreaks affecting the majority of animals. In both situations, however, the seriousness of infection depends on the number of organisms present in the glands and the species of yeast involved (Pengov, 2002). Generally, studieshave shown that mycotic mastitis is on the increase and the most frequently isolated organisms from affected quarters are *Candida* species (Malinowski *et al*., 2001; Spanamberg *et al*., 2008; Tarfarosh and Purohit, 2008) which are a group of unicellular opportunistic organisms, ever present in the natural surroundings of dairy cattle (milker's hands, milking machines, treatment instruments, floor, straw, feed, saw dust, soil, drug mixtures and sanitizing solutions) and are normal inhabitants of the skin of the udder and teats, where they exist in low numbers (Santos and Marin, 2005). They can invade mammary glands and cause clinical mastitis characterized by pain, prolonged fever, tenderness, inflammatory reaction in the mammary gland and associated lymph nodes,and also,

reductions of milk yield and quality in animals (Şeker, 2010). Some intra-mammary fungal infections such as *A. fumigatus* and *Candida* spp. may result in death of affected animals (Krukowski *et al*., 2000).

Outbreaks of mycotic mastitis are generally believed to result from an ascending infection subsequent to incorrect administration of antibiotic preparations during drying-off period (Spanamberg *et al*., 2008),contamination of the teat end or cannulas by environmental yeasts and fungi, lack of hygiene during milking and poor equipment cleaning(Gaudie *et al*., 2009). Also, administration of large doses of antibiotics may cause a reduction in vitamin A leading to injury to the udder's epithelium and affecting the micro-flora of the mammary glands, which acts as an animal natural defense, thus facilitating the invasion of fungi and yeasts (Şeker, 2010).

The clinical signs of mycotic mastitis are non-specific and in some cases, their development may be masked by symptoms of an underlying disease. Therefore, the disease is generally diagnosed by demonstrating and identifying the etiological agent histopathologically and in culture (Krukowski *et al*., 2000; Santos and Marin, 2005).

Current diagnostic methods have not proven to be sufficiently sensitive and specific to enable an early and effective diagnosis of the disease, with the result that the search for an optimal diagnostic method continues (Garcia and Blanco, 2000). Among non-culture methods under investigation, Polymerase Chain Reaction (PCR) offers advantages over classical approaches, because theoretically, low level fungal infections (e.g. with *Candida albicans*) can be detected from minimal volumes of clinical samples such as blood, and DNA from both dead

and viable organisms could serve as a target template for the amplification reaction (Polanco *et al*., 1999).

Random use of growth promoters, antibiotics and antimycotics in animals leads to immune suppression and development of multiple drug resistant strains to compounds used in human medicine (Ström *et al*., 2002; Magnusson *et al*., 2003). Probiotic bacteria present in the alimentary tract and vagina of humans and animals prevent the overgrowth of *Candida* spp. and thereby decrease the occurrence of mucosal or systemic candidiasis (Drozdowicz and Kwiecien, 2006; Zwolinska-Wcislo *et al*., 2006).The increasing incidence of mycotic infection, the increasing resistance of mycoticspecies to antifungal agents and the rise in mortality associated with infections by *Candida* species demand a safe way to prevent and treat infections such as mycotic mastitis caused by opportunistic yeasts and fungi.

Therefore, the present study reports mycotic mastitis in some peri-urban dairy farms in Kaduna State, Nigeria, and the causal mycotic agents, diagnosed directly using nested PCR and DNA sequencing. This study draws attention to the importance of the disease in Nigeria, and could therefore stimulate interest for further investigations.

1.2 Statement of Research Problem

Mastitis is one of the most serious problems in the dairy cattle farms. The great majority of cases are caused by bacteria, but recently there have been an increasing number of reports about cases caused by yeasts or yeast-like organisms, following post-treatment with antimicrobial agents (Crawshaw *et al*., 2005; Saleh, 2005).Studies have shown that fungal organisms and their spores can survive pasteurization and that yeast intra-mammary

infections were responsible for 2-3% of all clinical cases seen in a veterinary practice (Chahota *et al*., 2001; Spanamberg *et al*., 2008).

Nowadays, public health concern associated with microbial food safety has arisen. Numerous epidemiological reports have implicated non-heat treated milk and raw-milk products as the major factors responsible for illnesses caused by food-borne pathogens (Oliver *et al*., 2009). Some yeast may further pose threats to food safety given their association with opportunistic infections and other adverse conditions in humans (Fleet, 2007).

There are few documented cases of mycotic bovine mastitis in Nigeria prior to this study. In all forms of mastitis, a cow may not reach her full production potential as some of the milk secretory tissue would have been destroyed. Yeast infections should be suspected when there is a history of unsuccessful treatment or an intensification of clinical signs of mastitis after intra-mammary infusion of antibiotics (Krukowski *et al*., 2006). Although antimycotic drugs have been used for treatment of yeast mastitis, there was no clear evidence of the effectiveness of this therapy (Crawshaw *et al*., 2005).

1.3 Justification of the Research

Mastitis is the most widespread infectiousdisease of cattle and from economic aspect the most damaging (Dodd, 1985; Oliver*et al*., 2009). However the rate of subclinical mastitis is 15 to 40 times greater than the clinical cases (National Mastitis Council (NMC), 2000).

The normal somatic cell count (SCC) is 200,000 SCC/ml of milk, however the cow is not considered to be infected until the count rises to 300,000 SCC/ml of milk (NMC, 2000). Each

time the count rises by 100,000 SCC/ml of milk, the loss of milk production is about 2.5% per lactation. For example, for a herd that has a cell count of 400,000 SCC/ml, the loss of milk due to mastitis would be 5%. At the same time as the SCC increases, the yield of processed products such as yoghurt and cheese will decline by 1%. Reducing SCC is of benefit not only to the farmer, but to the processor as well. Increased mastitis prevalence can be expected with increased milk production unless all lactating cows are tested at least monthly and treated during the dry period (Burns *et al*., 2000; NMC, 2000; FAO, 2008).

Mastitis presents grave public health hazards for the human populace as milk is a good vehicle for disease transmission to humans when inadequately treated. Some of the important milkborne diseases includes; candidosis (thrush), tuberculosis, Q-fever, brucellosis, salmonellosis, botulism, campylobacteriosis, *Escherichia coli*, diptheria, hepatitis A, histamine intoxication, Iron intoxication, milk sickness, shigellosis, staphylococcal intoxication, streptococcal sore throat, scarlet fever, etc. (El-Sharoud *et al*., 2009; WHO, 2010).

The increasing number of dairy farms coupled with the presence of mycosis in animals may be an indicator of its probable presence in man especially in those working with cattle or their by-products or consuming unprocessed milk and milk by-products. In most parts of the northern states of Nigeria, local customs encourage the consumption of raw bovine milk with public health safety problems (Ocholi *et al*., 2004).

There are increasing reports of failure of intra-mammary antibiotic treatment in bovine mastitis by farmers and veterinarians in Nigeria and Northern States in particular (Kwaga, 2012).This study was designed to provide information on fungal and coliformmicro-flora

ofbovine milk. The *in vitro* susceptibility of the isolates to various antimicrobials agents was evaluated to provide comprehensive information on bovine mastitisin Kaduna metropolis, Kaduna State, Nigeria.

1.4 Aim of the Study

The aim of the study was to determine the diversity and prevalence of yeasts and coliforms associated with bovine mastitis in peri-urban dairy farms in Kaduna State, Nigeria and employing molecular techniques in yeast identification.Alsothe risk factors associated withbovine mastitis and the antibiograms of isolates.

1.5 Objectives of the Study

The objectives of this work are to:

i. establishthe occurrence of subclinical mastitis in cows by using the California mastitis test (CMT).

ii. culture, isolate and identify yeasts and coliform organisms from milk of cows with subclinical and clinical mastitis in Kaduna metropolis using API 20C-AUXand Microgen GN-ID A+B Kit (Medica-Tec^{TM}).

iii. determine the *in vitro* susceptibility of the isolates to various antimycotic and antibioticagents.

iv. characterize the yeasts associated with milk of cows using polymerase chain reaction (PCR) and sequencing of the domains D1 and D2 of the 26S rRNA gene.

v. establish association between various predisposing factors of subclinical mastitis (age, parity number, stage of lactation, management practices, teat/udder injury) and

occurrence of mastitis in cows using structured questionnaires.

1.6 Research Questions

1. Are there cases of subclinical mastitis in cows in Kaduna metropolis?
2. What are the different types of yeast and coliformorganisms in milk of cows with mastitis in Kaduna metropolis?
3. What antimycotic and antibiotic agents are the isolates susceptible to?
4. Areyeasts of public health importance present in milk from cows with mastitis in Kaduna metropolis?
5. Are there any associations between the risk factors of subclinical mastitis caused by coliform organisms?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Synonyms for Mastitis

Mastitis is a very common disease worldwide and every tribe has a name for describing the condition.These are some of the names it is known as across the world: Tuo thuno(Luo); yele(Embu); hiha goru(Gabbra); miatapkinai(Kipsigis); lifuuti(Maragoli); candhobarar, candabarar, carar, galleh, goof(Somali); loebeta(Turkana); Murimu wa nyundo, kuruara thukamo(Kikuyu); Enkeeya Ollki(Maasai); mammitis, mammite(French); inflammation de la ubres(Spanish); *Dagadi*(Fulani); ciwon kumburi nono (Hausa)(Intermediate Technology Development Group and International Institute of Rural Reconstruction (ITDG and IIRR),1996).

2.2 Some Important Uses of Cattle

Cattle (colloquially cows) are the most common type of large domesticated ungulates. They are a prominent modern member of the subfamily *Bovinae*, are the most widespread species of the genus *Bos*, and are most commonly classified collectively as *Bos taurus* (Radostits*et al*., 2007; Bollongino *et al*., 2012). Cattle are raised as livestock for meat (beef and veal), as dairy animals for milk and other products, and as draft animals (oxen / bullocks) pulling carts, plows and the like. Other products include leather and dung for manure or fuel. In some countries, such as India, cattle are sacred (cattle have significant religious meaning). It is estimated that there are 1.3 billion cattle in the world today (Cattle Today, 2013). In 2009, cattle became the first livestock animal to have a fully mapped genome (Brown and David, 2009). Some consider cattle the oldest form of wealth, and cattle rustling consequently one of the earliest forms of theft.

2.3 Importance of Milk

Milk is defined as that fresh, clean and normal mammary secretion obtained by milking of one or more dairy cows that are properly fed and kept (Radostits *et al*., 2007). Although several types of milk are known (e.g. human milk, donkey milk, goat milk, camel milk), globally cow milk is a good source of animal protein, fats, vitamins and minerals to the human body. In addition, nutritionally less useful substances like enzymes are also present in normal milkused as indices in screening or quality control tests (Burns *et al*., 2000; Oliver *et al*., 2005). The obvious need for milk hygiene and thorough milk inspection before human consumption can therefore not be over emphasized, since in certain diseases affecting the udder of the host, the milk could act as a vehicle for the disease causing agents to be transmitted to the consumer (Murphy and Boor, 2003).

2.4 Mastitis

Mastitis can affect all species and breeds of animals including humans that are lactating. Mammary gland function is important for the health and growth of the newborn animal, but udder diseases have been documented to have a negative effect on both factors and can pose public health hazards for populations consuming milk as fresh, raw, soured or processed into cheese (Kudi *et al*., 1997).Mastitis is responsible for heavy financial losses to the dairyman due to discarding of abnormalmilk, reduced milk production and butter fat, decreased market value of cow and cost of drugs and veterinary services (Ameh *et al*., 1999; FAO, 2008). Mastitis occurs wherever and whenever the animals are bred, fed and managed with the intention to increase milk supply. Indeed the higher the level of production, the higher the occurrence unless there is very strict control measures continuously put in place. The animal

is predisposed to the disease by several factors such as the age, state of lactation, milk yield, hereditary factors, trauma and poor milking hygiene (Sibtain *et al*., 2012).

Mastitis takes two forms: clinical mastitis, which is easy to diagnose because symptoms are visible, and subclinical mastitis where symptoms are invisible and require indirect means of diagnosis (Radostits *et al*., 2005).

2.5 Mycotic Mastitis

Mastitis in cows is caused by about 150microorganisms, many of which are pathogenic for humans (Radostitis *et al*., 2007). The most common aetiological factors arebacteria followed by mycoplasmas, viruses, fungi, andalgae (Malinowski *et al*., 2001; Malinowski *et al*., 2002;Wellenberg *et al*., 2002; Malinowski *et al*., 2006). Fungi are widespread in nature, being noted inbedding and gear from the stables, on milking machines.The studies on fungalinfections of the mammary gland in cows areincreasingly common due to their growing incidence (Kivaria and Noordhnizen, 2007).According to literature, data on such infections account for2–13% of all cases of mastitis in cows (Lagneau *et al*., 1996; Krukowski *et al*., 2000; Krukowski *et al*., 2006)sometimes, their incidence is much higher or they areenzootic. The species of fungi that may be involved in the pathology of mycotic mastitis in cowsare: *Cryptococcus neoformans, Trichosporon* spp., *Candida* spp., *Saccharomyces* spp.,*Pichia* spp., *Torulopsis* spp., *Aspergillus* spp., *Rhodotorula* spp., *Geotrichum candidum,Trichoderma koningii, Trichotecium roseum;* amongst them *Candida* spp., isfound most frequently (Lagneau *et al*., 1996; Keller, 2000; Krukowski *et al*., 2000;Malinowski and Kłossowska, 2002; Santos and Marin, 2005; Krukowski *et al*., 2006; Kivaria and Noordhnizen, 2007).However, *C. neoformans* and *C. albicans* are the most

common pathogenic organisms of bovine mastitis (Krukowski *et al*., 2000;Malinowski and Kłossowska, 2002). The incidence of mastitis due to yeasts is usually low in dairy herds, but sometimes it can occur in epizootic proportions. Teat injuries may predispose to the establishment of a yeast infection (Gonzalez, 2001; Jand *et al*., 2003; Spanamberg *et al*., 2009).

Yeasts are known to cause diseases in both man and animals which include thrush, disseminated candidosis, cryptococcosis, mastitis (Malinowski and Kłossowska, 2002; Asfour *et al*., 2009). Thelikely sources of infection are through the administration of contaminated intramammary infusions or from other infected cows. The excessive use of antibiotics, corticosteroids, immunosuppressive drugs as well as chronic diseases are the major contributing factors in increasing the incidence of diseases caused by yeasts (Jand *et al*., 2003; Donskey, 2004; Das and Josef, 2005).

Fungal infections can occur in cervicovaginal cavity of Holstein dairy cows with or without reproductive diseases butno epidemiological risk ispresent when the milk is heat treated before being consumed, as this yeast does not tolerate thepasteurization temperature of milk (Garoussi *et al*., 2007; Kivaria and Noordhnizen, 2007). Although *Candida* spp. is a normal commensal of oral mucosa, increased exposure due to proximity to affected animals and or consumption of contaminated milk could be the cause for development of thrush in milkers (Oliver*et al*., 2005). *Candida* spp. infection is often acuteand transient, theorganism disappearing in about three weeks but, in the infections with *Aspergillus fumigatus* and*Aspergillus nidulans,*numerous abscesses surrounded by granulation tissueform in themammary quarters, although the lactipherous ducts are generally not affected(Keller *et al*., 2000; Smith and Hogan, 2001; Matofari *et al*., 2003).

Fungi are a group of unicellular organisms widely distributed in nature as normal flora of the soil and may colonize udder skin, being noted inbedding and gear from the stables, also, on milking machinesbut only the yeasts are usually implicated as the cause of mycotic mastitis in dairy animals (Radostits *et al*., 2005; Kivaria and Noordhuizen, 2007). Yeasts may gain entrance into the mammary gland of dairy cows from the environment carving their way through teat injuries or tissue damage caused by a previous bacterial infection, or may follow intra-mammary treatment (Gonzalez, 1996; Malinowski and Kłossowska, 2002).

Majority of yeasts are considered saprobic, potentially pathogenic and opportunists as they produce disease when natural defense mechanisms are lowered with the most frequent genus being *Candida* (El-Sharoud *et al*., 2009). Other genera like *Cryptococcus, Pichia* and *Trichosporon*, albeit found at low prevalence, have also been isolated from clinical mastitis cases (Krukowski *et al*., 2000; Krukowski and Saba, 2003; Kurtzman *et al*., 2011).

Candida is commonly viewed as an opportunistic yeast pathogen. In the normal host, the yeast has evolved to become a successful commensal. It expresses variant traits critical for existence on mucosal surfaces. In the abnormal host, the same traits become virulence characteristics accounting for invasive abilities as the delicate balance of *Candida* with the host shifts in favour of the yeast (Santos and Marin, 2005).

Literature have shown the occurrence of yeasts in milk and cheese processed from milk derived from bovine, buffalo, camelidae, ovine and caprine species (Corbo *et al*., 2001; Kalla *et al*., 2008; El-Sharoud *et al*., 2009). The environmental contamination associated with lack of hygiene during milking and poor equipment cleaning has been implicated in the development of mastitis (Kivaria and Noordhuizen, 2007).

It is thought that, the inappropriate use of drugs such as over-use, over-prescription, improper and excessive use of antibiotics in livestock and feeds and indiscriminate use of broad-spectrum drugs (Kwaga, 2012; WHO, 2010) might be factors that propitiate the occurrence of mycotic mastitis, because they affect the microflora of the mammary glands, which act as natural defense of the animals (Kalla *et al*., 2008; Junaidu *et al*., 2011). It is well established that the yeasts of the genus *Candida* are capable of utilizing antibiotics like penicillin and tetracycline as nitrogen sources (Spanamberg *et al*., 2008). In addition, large doses of antibiotics may cause reduction in the vitamin A, leading to injury to the epithelium of udder, facilitating the invasion of yeasts (Krukowski *et al*., 2000). Other sources of yeast infection have been traced to the use of yeast contaminated antimicrobial preparations (Spanamberg *et al*., 2004; Fleet, 2007).Moreover, the virulence of microorganisms andimmunosuppression of animals are relevant (Wawron and Krzyżanowski, 2002; Staroniewicz *et al*., 2007; Spanamberg *et al*., 2008).

Studies have shown that incidence of mycotic mastitis was usually very low in dairy herds in the developed countries. For instance, in the United States, about 2-7% (Kirk and Bartlett, 1986), in Belgium constitutes less than 6% (Lagneau *et al*., 1996), in Italy 4.4% (Moretti *et al*., 1998), and in Denmark 1.4% (Aalbaek *et al*., 1994).A study to assess the incidence of mycotic mastitisin the middle-eastern part of Polandshowed 7.1% of 2,122 milk samples from cows with clinicaland subclinical mastitis positive for yeasts (Władysław *et al*., 2010).

In a study carried out in Brazil on 2,078 milk samples from healthy and mastitic animals, 10% of the isolates were yeasts, with 3.2% being of the genus *Candida* (Costa *et al*., 1993). In Southern Brazil, *Candida* species was isolated in 1.3% of 896 samples of infected animals,

among which 0.9% were *C. albicans*. In the State of São Paulo, *C. albicans* was isolated in 8.9% amongst 260 milk samples from cows with mastitis (Santos and Marin, 2005).

In Egypt, 6.1% and 6.5% prevalence rates were reported in cattle and buffalo respectively (Awad *et al*., 1980). Other reports are, 17.0% in dairy herds in Tanzania (Kivaria and Noordhuizen, 2007) and 10.2% from camels in Kano state, Nigeria (Kalla *et al*., 2008). Various other studies have reported the presence and isolation of yeasts in milk samples from animals with mastitis (Santos and Marin, 2005; Krukowski *et al*., 2006) and also from storage tanks for milk (Spanamberg *et al*., 2004).

Mycotic mastitis is an important disease from public health standpoint as the presence of different fungi in milk renders it unsuitable for human consumption and helps in the spread of diseases like; thrush, candidemia andcryptococcosis. (Oliver *et al*., 2005; FAO, 2008; WHO, 2010; Kurtzman *et al*., 2011).

The prevalence of mycotic mastitis in Nigeria is not accurately known although there have been some studies, but, research has been very limited on the dynamics of bovine mycotic mastitis pathogens (Kalla *et al*., 2008; Junaidu *et al*., 2011).

2.6 Diagnosis of Mycotic Mastitis

Diagnostic mycology largely rests on a detailed study of morphological evaluation of the isolates and has therefore been termed as “exercise in contemplative observation” (Ananthnarayan and Panikar, 2004).

Yeast colonies grown on blood agar at 37°C for 24-48 hours may be confused and misidentified as staphylococci or micrococci. The colonies of *Candida* species generally are opaque, often white or yellowish, and at first usually smooth. Their texture is creamy or pasty, and in a microscopic smear appear to consist solely of oval to round budding blastospores. *Cryptococcus neoformans* also grows well on blood agar at 37°C usually forming colonies within 48-72 hours. Colonies initially are pale and pasty, becoming honey-brown and mucoid later. Yeast is Gram-positive, but larger than bacteria and morphologically different when compared with bacilli and cocci (Gonzalez, 1996).

However, it is becoming evident that these tests are not adequate for the delineation and identification of yeast species (Prillinger *et al*., 1999) because they show high variability and cannot be relied on for proper identification of yeasts (Barnett *et al*., 2000). The application of molecular techniques to the identification of yeasts has provided a more useful tool for studying the biodiversity of yeasts in foods (Prillinger *et al*., 1999; Fleet 2007). These methods involve the analysis of the coenzyme Q system and the monosaccharide pattern of cell walls, random amplification of polymorphic DNA (RAPD), microsatellite analysis, restriction fragment length polymorphism (RFLP) of transcribed and spacer sequences of ribosomal DNA, chromosome polymorphism determined by pulsed-field gel electrophoresis (PFGE), and sequencing of the 18S rRNA and 26S rRNA genes. Some of these methods have been successfully used for the identification of foodborne yeasts (Kurtzman *et al*., 2003; Fernandez-Espinar *et al*., 2006; El-Sharoud *et al*., 2009).

The conventional microbiological methods such as culture are time-consuming, lack sensitivity and specificity and prone to the appearance of false negatives (Kawazu *et al*., 2004). Also the culture may be hazardous for the health of laboratory personnel (Mirhendi *et*

al., 2001). In all cases the growth is very difficult to interpret as truly etiological in the process. Because of its ability to directly detect extremely small quantities of DNA with high level of sensitivity and specificity, PCR technology offers potentially faster detection of infection and permits prompt treatment with antifungal therapy which will improve survival and reduce morbidity. It allows researchers to detect and identify fungal species in swabs, fluids and tissues (Pham *et al*., 2003; Shin *et al*., 2003; Scheuller *et al*., 2004; Abd El-Razik *et al*., 2011), in food as contaminants (Zur *et al*., 2002; Millar *et al*., 2003), also in indoor air, water, soil and building surfaces (Haugland *et al*., 2004). The most important step in the detection of yeasts and fungi using PCR is the ability to efficiently extract DNA from hyphae and or conidia. This step is even more critical when attempting to detect small quantities of mycotic material in biological samples such as blood (Garcia *et al*., 2004), mucus, milk (Abd El-Razik *et al*., 2007, 2008 and 2010) and tissues (Karakousis *et al*., 2006; Abd El-Razik *et al*., 2011).

2.7 Prevention and Treatment of Mycotic Mastitis

Sometimes, heifers that have never received intramammary infusions may develop yeast mastitis. If mastitis due to yeast is suspected, antibiotic therapy should be stopped immediately. Yeast or other mastitis infections can be reduced if the tip of the plastic infusion tube is only partially (rather than completely) inserted through the teat canal during intramammary therapy (O'Neil, 2006).

Therapy is given on the premise that treatment costs will be outweighed by production gains after elimination of infection. Drug distribution after intramammary administration may not be adequate because of extensive fibrosis and microabscess formation in the gland; it is

critical to assess the cow's immune status from a perspective of duration of infection and number of quarters infected(Crawshaw *et al*., 2005).

Although antimycotic drugs have been used for treatment of yeast mastitis, there is no clear evidence of the effectiveness of this therapy (Spanamberg *et al*., 2004; Fleet, 2007)

2.8 Coliform Mastitis

Almost any microbe that can opportunistically invade tissues and cause infection can cause mastitis. However, most infections are caused by various species of streptococci, staphylococci, and gram-negative rods, especially lactose-fermenting organisms of enteric origin, commonly termed coliforms. *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae* and*Serratia marcescens* are four common coliform bacteria that cause mastitis (Junaidu *et al*., 2011).

Coliform bacteria are normal inhabitants of soil, digestive tract and manure.They accumulate and multiply in contaminated bedding. Coliform numbers of1,000,000 or more per gram of bedding increase the likelihood of an udder infectionand clinical mastitis. *Klebsiella pneumoniae* is common in sawdust bedding,especially rough-cut sawdust that contains bark or soil (Kagkli *et al*., 2006).

Coliforms invade the udder through the teat sphincter when teat endscome in contact with coliform bacteria. Once coliform bacteria enter the mammarygland, they either multiply rapidly or remain dormant. However, the immune response of the cow is highly successful in destroyingthese bacteria. Many inflammatory and systemic changes seen in severe coliform

mastitis result from the effects of release of lipopolysaccharide (LPS) endotoxin (a component of the bacteria cell wall) and subsequent activation of cytokine and arachidonic acid–derived mediators of inflammation and the acute phase response. By the time therapy was initiated, maximal release of LPS had occurred (Burns *et al*., 2000; Smith and Hogan, 2001).Coliform bacteria are responsible for a great number of acute clinical mastitiscases in dairy cows. Severely affected cows may show signs of high fever,udder inflammation (swelling), depressed appetite, dehydration (sunken eyes),diarrhea, decreased production and abnormal milk. Coliform bacteria are also capable of producingsubclinical infections that persist for longer periods of time (Radostits*et al*., 2007).

Studies have shown that mastitis caused by coliformsmay result toagalactiaand therefore cause substantial losses to producers (NMC, 2000). This disease costs the US dairy industry about 1.7 to 2 billion USD each year(Jones and Bailey, 2010). Consequently, the economic losses resulting from infection, the relatively high incidence of infection as compared to other clinical mastitis pathogens, and the occasional severe nature of infection, puts coliform mastitis to be a major problem confronting dairy industry (FAO, 2008; Junaidu *et al*., 2011).

Mastitis caused by coliforms results in a higher incidence of cow deaths or agalactia-related culling (30–40%) than mastitis caused by other pathogens (2%). Prognosis for cases of *Klebsiella* infection should be particularly guarded, because these cows are twice more likely to be culled or die than those infected by other coliforms(Jones and Bailey, 2010). *Serratia* mastitis may arise from contamination of milk hoses, teat dips, water supply, or other equipment used in the milking process. The organism is resistant to disinfectants(Kagkli *et al*., 2006).

The bedding used to house cattle 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 (Matofari *et al*., 2003; Kivaria and Noordhuizen, 2007). A study carried out in Sokoto State, Nigeria revealed *E. coli* (9.78%), *Klebsiella* spp. (4.35%), *Proteus* spp. (8.69%)and *Enterobacter* spp. (1.09%)(Junaidu *et al*., 2011).A cross-sectional study carried out in Gondar town, Ethiopia had 54 different bacterial species identifiedbut,*E. coli* (29.6%), *Pseudomonas aeruginosa* (18.5%), and *Klebsiella pneumoniae* (16.7%), were the most commonly identified gram-negative staining bacterial pathogens (Garedew *et al*., 2012). Majority of coliformisolates from a study conducted using the raw milk consumed in Khartoum, Sudan were *E. coli* 32%, *Enterobacter*spp. 29.2%, *Klebsiella* spp. 19.4%, *Serratia* spp. 11.1% and Citrobacter 1.0% (Salman and Hamad, 2011).

2.9 Diagnosis of Coliform Mastitis

A clinical diagnosis of coliform mastitis is usually based on clinical signs, culture of coliform organisms from milk, and a high somatic cell count. In many cases, culturing for coliform bacteria may be unrewarding because host defenses can clear the bacteria from the gland in one to two milkings. Streaking blood agar and MacConkey agar plates with 0.01 ml and 0.1 ml of milk, respectively, may aid in obtaining detectable growth and a definitive diagnosis. If the animal has already succumbed to the effects of endotoxemia, bacteriology of chilled mammary tissue and regional lymph nodes along with histologic evaluation of formalin-fixed mammary tissue are diagnostic in confirming coliform mastitis (Radostits *et al*., 2007; Pyorala, 2008).

2.10 Prevention and Treatment of Coliform Mastitis

Prevention of coliform mastitis is difficult because *Escherichia coli*, *Klebsiella*, and *Enterobacter* are ubiquitous even in the most well managed dairies. Culturing individual milk samples is important to identify the major pathogens in the herd. Improving pre-milking hygiene and frequent evaluation of milking machine function may reduce bacterial load and teat end changes. Proper administration of dry cow intramammary antimicrobials and teat sealants also reduces existing bacterial infections while preventing new infections during the dry period. Vaccination with a core lipopolysaccharide antigen vaccine (J5 mutant *E coli*) may also reduce the severity of coliform mastitis (Blowey and Edmondson, 2010; Quesnell, 2012).

Even though the bacterial invasion begins in the mammary gland, treatment of coliform mastitis must also combat the systemic endotoxemia. Broad spectrum intravenous antimicrobials are given to severely affected cows to reduce the bacteremia which may be present in up to 48% of coliform mastitis cases. Intramammary infusions of antibiotics typically do not affect the outcome of coliform mastitis cases but may be beneficial in decreasing pathogenic gram-positive organisms within the mammary gland. Stripping of the affected quarter several times a day, in conjunction with oxytocin injections, has also been utilized to reduce the number of bacteria and endotoxin in the gland. Anti-inflammatory and fluid therapy may also be necessary to prevent shock and maintain overall hydration status(Pinzon, 2011; Quesnell, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Kaduna State is one of the seven states (Sokoto, Kebbi, Zamfara, Katsina, Kano, Jigawa and Kaduna) in the Northwest geopolitical zone of Nigeria.It is located on Latitude (DMS) 10.3333 N and Longitude (DMS) 7.7500E(Figure 3.1). Kadunawas the capital of the then Northern region of Nigeria, having 23 Local Government Areas (LGAs) with three Senatorial Districts. The State is bordered in the Northwest by Niger and Zamfara States, on the Northeast by Katsina and Kano States. It also shares boundaries with Niger State on the West, Bauchi and Plateau states on the East and, on the Southern end with the Federal Capital Territory, Abuja and Nassarawa State.

The cattle population in the State as at 1992 was 1,006, 634 (RIMS Report, 1992). There has been no recent survey since then but the State Ministry of Agriculture estimates an annual increase of 1.5%, therefore, there should be about 1,500,000 million cattle presently. The White Fulani (*Bos indicus*) breed makes up about 90% of this cattle population. Extensive husbandry system, where the pastoralists move around with their animals in search of green pasture is widely practiced. Kaduna State is in the savannah ecological zone, which is characterized by equal duration of wet and dry seasons, high annual temperature (average) of the range 28-32°C, few scattered trees and grasses, gentle slope and large farm holdings. Maize, sorghum, millet, wheat, rice, cowpea, pepper and onion are the major crops that thrivein savannah.

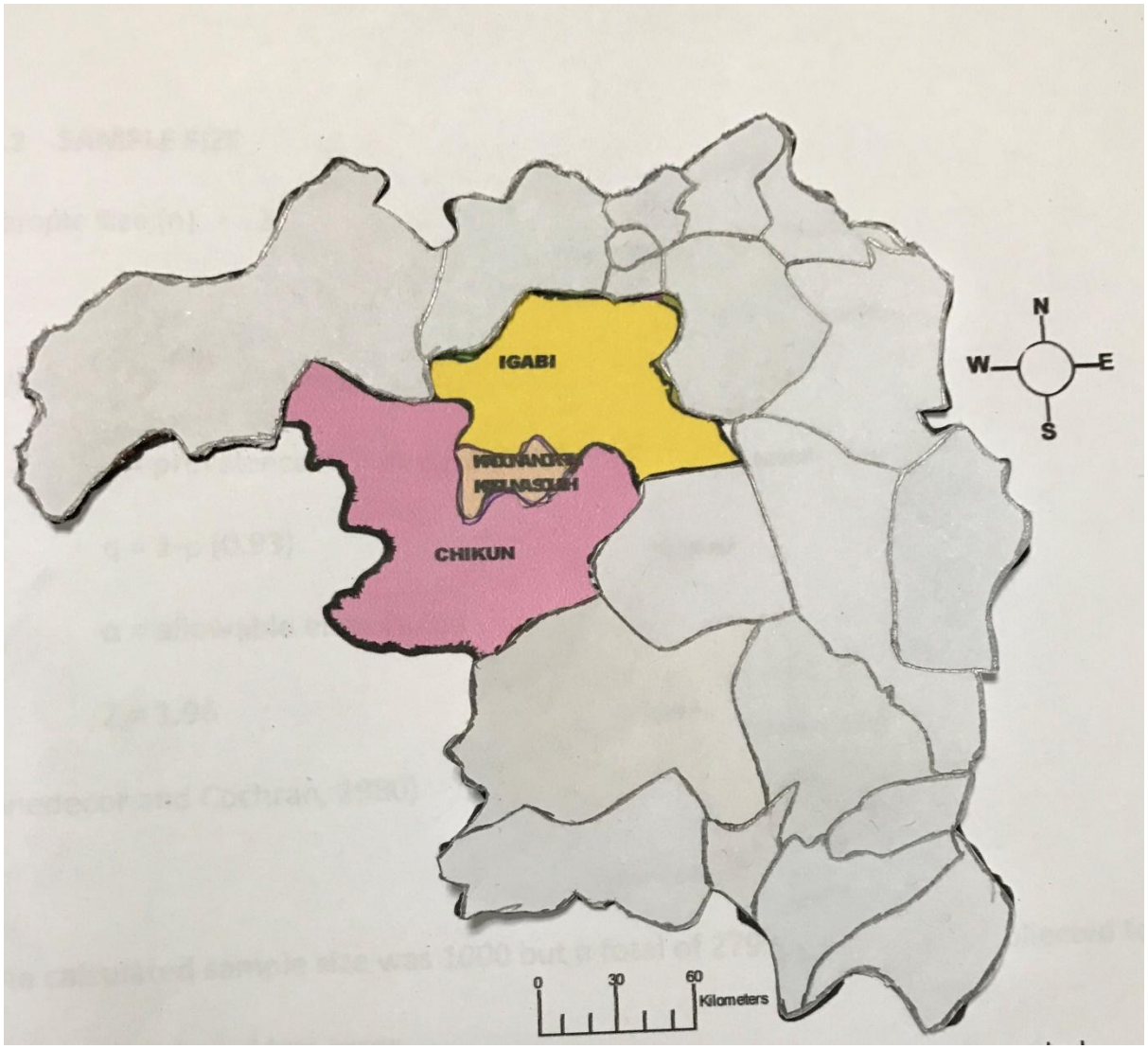

Fig 3.1: **Map of Kaduna State showing the four Local Government Areas where cows weresampled.**

Igabi

Kaduna north and south

Chukun

LGAs not sampled

3.2 Sample Location

Samples were collected from four LGAs that make up Kaduna metropolis, Kaduna State namely; Chikun, Igabi, Kaduna north and Kaduna south.

3.3 Sample Size Determination

The samplesize for this cross-sectional studywas determined using the formula as described by Snedecor and Cochran, (1989).

$$\text{Sample size (n)} = \frac{(Z_{1-\alpha})^2\left(P\ (1-P)\right)}{D^2}$$

Where: CI = 1-α = 95%

$Z_{1-\alpha} = Z_{0.95}$ =1.96 [Standard for Confidence Interval (CI) of 95%]

P = 0.102 prevalence of a disease from previous study (Kalla *et al*., 2008)

D = 5% = 0.05 (absolute precision required)

Substituting: $n = \frac{1.96^2 \times (0.102\ (1- 0.102))}{0.05^2} = 140$

The calculated minimum sample size (n) was **140** but a total of **300**pooled (of all quarters) milk samples were collected.

3.4 Selection Criteria

A cross-sectional study was carried out on farms in Kaduna state targeting peri-urban farms that provide milk to the community. Selection of farms was based on; different management conditions, willingness of the farms/pastoralists to participate in the study, and accessibility ofthe location, so that samples collected could be immediatelytransferred to the Laboratory for further analysis.All cows in-milking were sampled in every farm visited. It was ascertained that the cows did not receive any antibiotic treatment before sampling.

3.5 Physical Examination of Udder and Mastitis Screening Test

Individual animals were properly restrained, identified and clinicallyexamined. Briefly,clinical findings like abnormalities of udder secretions, abnormalitiesof udder size, consistency, and temperature of mammarygland were examined by visual inspection andpalpation. Pain reaction upon palpation, changes in themilk (blood tinged milk, watery secretions, clots, pus),and change in consistency of udder were considered asevidence of clinical mastitis.

Cows,which did not have clinical mastitis, weresubjected to further investigation for subclinical mastitisby using CMT onmilk samples from each quarter of sampled animals. Itwas carried out by adding equal amounts of CMTreagent and milk from each quarter on test paddle andwas rotated for 10 seconds. Samples with a CMT score of 0 or T(trace) were considered negative while those with CMTscores of 1 (mild clumping), 2 (moderate clumping), or 3(heavy clumping) were considered as positive. All the milk samples (300) were subjectedto culture.

3.6 Sample Collection

Afterwards, the teatswere disinfected using a disposable paper towel immersed in 70% ethyl alcohol, the first streamof milk (foremilk) was discarded before volumes of about 10ml (pooled) of all quarters were aseptically collected into labelled sterile universal bottles.The samples were kept in a cool box containing ice packs and transported to the Bacterial Zoonosis laboratory, Veterinary Public Health and Preventive Medicine Department, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.

3.7 Laboratory Isolation and Identification of Fungi

One milliliter ofthe milk sample to be tested was directly inoculated ontoSabouraud dextrose agar and evenly spread out with an 'L' shaped sterile glass rod (Darling).Plated culture media were incubated atroom temperature for up to 72 hours, and then colonial morphological identification and enumeration of counts were carried out thereafter. This was followed by staining of fungal colonies on glass slides with lactophenol cotton blue according to the method of David (2011).Growths on culture media were counted and expressed in standard form, in colony forming units per milliliter (CFU/ml) of the sample in consideration.

3.7.1 Purification of fungal colonies

All fungal isolates were subcultured into SDA plates containing gentamicin and incubated at room temperature for 72 hours. Thereafter, individual colonies were picked and stored on SDA slants in McCartney bottles until ready for use (El-Sharoud *et al*., 2009).

3.8 Morphological Studies of *Candida albicans*

3.8.1 Chlamydospore production

A plate of cornmeal-tween 80 (Chlamydospore agar) was inoculated bylightly touching a colony of freshly grown yeast with a sterile platinum loop then, using the loop to make an'X' cut in the medium. A thin glass coverslip was placed on the surface of the agar and the plates were incubated at 30°C for 2-4 days. The preparation was examined under low and high dry objectives for the thick walled chlamydospores borne on the tips of pseudohyphae (Zavalza-Stiker*et al*., 2006).

3.8.2 Germ tube test

Using a Pasteur pipette, 3 drops of fresh pooled human serum was dispensed into labelled 12 x 75 mm test tubes. With a sterile platinum loop, a yeast colony was lightly touched and suspended into the serum then, incubated at 35°C for 3 hours. Thereafter, a drop of the suspension was placed on a clean glass slide with a coverslip placed over the suspension and then, examined with a microscope using the low power objective. Thereafter a high power objective was used to confirm the presence (a young hypha growing out of a spore) or absence of germ tubes (Zavalza-Stiker *et al*., 2006).

3.9 Biochemical Tests for Fungi

The API 20C-AUX, acommercial yeast characterization kit (BioMerieux Vitek, France, product code 07221 C), was used according to the manufacturer's instructions for yeasts identification on the basis of metabolic / physiological features. The API 20 C AUX strip consists of 20 cupules containing dehydrated substrates which enable the performance of 19 assimilation tests. The cupules inoculated with a semi-solidminimal medium and the yeasts will only grow if they are capable of utilizing each substrate as the sole carbon source.The reactions were read after 24, 48 and 72 hours of incubation, by comparing growth in each cupule to the 0 cupule, which was used as the negative control. A cupule more turbid than the control indicates a positive reaction to be recorded on the result sheet and identification was obtained by referring to the identification software (Micheal *et al*., 1999).

3.10 Antifungal Sensitivity Testing

Antifungal sensitivity was carried out on all the isolates using commercial antimycotics (Oxoid). The antimycotics employed were: Fluconazole, Amphotericin B, Nystatin, Griseofulvin and Voriconazole.

Colonies of isolates to be tested were inoculated into peptone water andthe inoculum was standardized to 0.5 McFarland and incubated for 4hours. A sterile cotton wool swab was soaked into the peptone water culture and excess fluid expressed from the swab by rolling it on the inside wall of the culture bottle. The swab was used tostreak the entire surface of the SDA before placing antimycotic discs 15 mm apart. The plates were incubated at 35°C and read after 24 hours but for some strains where insufficient growth occurred, the plates were read after 48 hours.

The zones of inhibition were measuredin millimeters (mm) and compared against a reference standard which contains measurement ranges and their equivalent qualitative categories of susceptible/sensitive(CLSI, 2014).

3.11 Molecular Identification of Fungal Isolates

3.11.1 DNA extraction from fungal cells using phenol chloroform method

The following were dispensed into a labelled Eppendorf tube; 400µl lysis buffer, 48 hours old yeast isolate (picked using a plastic loop) and 25µl proteinase K, the tube was vortexed for 30 seconds and incubated for 2 hours (tubes were vortexed for 20 seconds every 10 minutes during the 2 hours incubation period). Thereafter, 400µl phenol chloroform was added, vortexed for 20 seconds and centrifuged at 13,000 rpm for 10 minutes.

The supernatant was pipetted into another labelled Eppendorf tube using one tip per sample and 400µl chloroform was added, vortexed for 20 seconds and centrifuged at 13,000 rpm for 5 minutes. The supernatant was pipetted into another labelled Eppendorf tube using one tip per sample then, 1000µl of 100% ethanol and 400µl sodium acetate were added then, the tubes were inverted and stored at -20°C overnight.

The tubes were removed from -20°C the next day, centrifuged at 13,000 rpm for 10 minutes at 4°C, the top clear layer was decanted then, 400µl of 70% ethanol was added to the sediment in the Eppendorf tube and centrifuged at 13,000 rpm for 5 minutes at 4°C then, decanted the top clear and spun for 2 minutes at 4°C.

Using one pipette tip per sample, the supernatant was carefully removed from the Eppendorf tube leaving the pellets behind. The pellets (genomic DNA) were air dried in the Sterilgard hood for 1 hour then; 50µl of Ultra-Pure Water (cell culture grade distilled water) was added to the pellets (genomic DNA) in the Eppendorf tube and stored at -20°C until ready to use as a DNA template for PCR amplification (Piotr and Nicoletta, 2006).

3.11.2 Reconstitution of primers

Based on the genomic nucleotide sequence of *C. albicans*two sets of gene-specific primers were designed and used for nested PCR amplifications for the identification of the genus*Candida*to thespecies level. The Sterilgard hood was wiped and disinfected using 70% ethanol in 10% bleach. All the 4 primers used for the PCR were centrifuged at 13,000 rpm for 30 seconds then, reconstituted by adding ultra-pure water according to the manufacturer's instruction in the hood.

Primer ITS1 + 111.5µl water

Primer ITS4 + 112µl water

Primer NL1 + 116.6µl water

Primer NL4 + 110µl water

The reconstituted primers were vortexed for 30 seconds and thereafter, each primer diluted by taking 10µl primer and 90µl water into labelled Eppendorf tube. (ITS = Transcribed spacer)

3.11.3 Polymerase chain reaction (PCR) amplification

The sample DNA was removed from -20°C storage, using a hot start premix tube, DNA samples were amplified in a reaction mixture (20µl) that contained 1µl genomic DNA + 1µl ITS1 + 1µl ITS4 + 17µl water. Positive and negative controls were included in the reaction (for the negative control, 1µl water instead of 1µl DNA was added) then, spun for 10 seconds. 1 drop of mineral oil (by Beckman coulter) was added to prevent evaporation then, spun again for 10 seconds.The hot start premix tubes were loaded into the PCR machine (Programmable Thermal Controller PTC-100^{TM}, MJ Research, INC, USA) and the following PCR cycle parameters were set according to, El-sharoud *et al*., 2009:

Step 1: 94°C for 7 minutes (denaturing of DNA molecules)

Step 2: 30 cycles of 45 seconds at 94°C (denaturation)

Step 3: 1 minute at 59°C (annealing)

Step 4: 30 seconds at 72°C (extension)

Step 5: 7 minutes at 72°C (final extension)

Amplification of the 5.8S-ITS rDNA region was achieved with the primers;

ITS1 (5□-TCCGTAGGTGAACCTGCGG-3□) and

ITS4 (5□-TCCTCCGCTTATTGATATGC-3□)(White *et al*., 1990).

At the end of the first round PCR, the PCR products were further amplified byusing another set of hot start premix tubes containing the following; 17µl water + 1µl NL1 + 1µl NL4 + 1µl first round PCR product. Then, spun for 10 seconds, 1 drop of mineral oil was added and spun for another 10 seconds.The second set of premix tubes were loaded into the PCR machine and the PCR cycle parameters were repeated.

Amplification of the D1/D2 domains of the 26S rRNA gene was achieved with the primers;

NL-1 (5□-GCATATCAATAAGCGGAGGAAAAG-3□) and

NL-4 (5□-GGTCCGTGTTTCAAGACGG-3□)(White *et al*., 1990). The expected size of the PCR products(amplicons) was 600bp. (Barnett *et al*., 2000; El-sharoud *et al*., 2009).

3.11.4 Loading samples and running an agarose gel electrophoresis

At the end of the second round PCR reaction, thePCR products were analysed by agarose gel electrophoresis in TBE (89 mMTris, 89 mMboric acid,2mMEDTA, pH 8.0) at 100 V for approximately1 hour in gel composed of 1.2% agarose (Agarose-ME, Classic Type) (Nacalai Tesque, Inc., Kyoto,Japan). Gels were stained with 0.5µg/ml ethidiumbromide (Nacalai Tesque, Inc.) in distilled waterat 21–25°C for 30 min, and then the gels weredestained at 21–25°C for 30 min in distilled water.

The gel electrophoresis apparatus was loaded with the second round PCR products as follows;

100 bp DNA ladder (marker) in lane 1

Second round PCR product of each sample in subsequent lanes

Second round PCR product of negative control after samples

Second round PCR product of positive control after negative control

The DNA bands were visualized with a UV transilluminator. Band sizes were estimated by comparison against 100 bp DNA ladder (marker)photographed and documented using the gel documentation system.The amplicon/band of interest (600 bp) was cut out and stored in Eppendorf tube at 4°C until ready to use.

3.11.5 PCR product extraction from gel

The cut out band of interest was removed from storage and weighed. To each 100mg of gel, 300µl of GB buffer was added. This was heated in the heat block for 10 minutes while inverting the tube every 3 minutes to make sure it melts completely.

All the contents in the heated tube was poured into labelled Hi-binding mini columns, centrifuged at 10,000 rpm for 1 minute, the flow through content was discarded then, 750µl PE buffer (wash buffer) was added, allowed to stand for 5 minutes and centrifuged at 13,000 rpm for 1 minute, the supernatant was discarded and 750µl PE buffer added.

It was centrifuged at 11,000 rpm for 1 minute and the flow through content discarded. Afterwards, centrifuged at 13,000 rpm for 2 minutes to get rid of all the ethanol in the PE buffer. The collection tube was discarded and replaced with a new tube.Thereafter, 20µl ultra-pure water was added and allowed to stand for 5 minutes,then, centrifuged at 13,000 rpm for 2 minutes. The entire DNA was emptied into a new labelled Eppendorf tube and

later, the DNA was loaded in the gel electrophoresis and ran at 100 volts for 20 minutes to check for bands.The DNA product was stored at -20°C until ready to use.

3.11.6 Purification of DNA products

Amplified PCR D1/D2 26S rDNA products were purified using E.Z.N.A.® Cycle-Pure Kit, OMEGA Bio-tek, Georgia, USA. Purification was done by removing the PCR product from -20°C storage, 100µl PB buffer was added and mixed using pipette tip. The mixture was transferred into Hi-binding columns and centrifuged at 10,000 rpm for 1 minute. The flow through was discarded and 650µl PE buffer added and allowed to stand for 3 minutes, then centrifuged at 13,000 rpm for 1 minute. The flow through was discarded and 650µl PE buffer added then, centrifuged at 13,000 rpm for 1 minute and discarded the flow through. The empty columns were centrifuged for 2 minutes; the collection tube was discarded and replaced with new tube.

10µl sample loading solution (SLS) was added to the matrix (center) column and allowed to stand at room temperature for 3 minutes then, centrifuged at 13,000 rpm. The elution step was repeated and spun immediately.

3.11.7 DNA sequencingand phylogenetic analysis

For the direct confirmation of specificity of thePCR products, the DNA fragments generated byeach of the primer pairs were sequenced.

Purified amplified PCR D1/D2 26S rDNA products were loaded into sample loading plate and directly sequenced using the E.Z.N.A.® Ultra-Sep Dye Terminator Removal Kit (OMEGA Bio-tek, Georgia, USA) by placing into a CEQ™ 2000XL DNA Analysis System Machine (Beckman Coulter, USA) for 2 hours. PCR primers NL-1 and NL-4 of the D1/D2 26S rRNA gene were used in the sequencing reactions to read both DNA strands.Sequences were edited and assembled using MEGA6 (Tamura *et. al.,* 2013)and then subjected to GenBank BLASTN (Yeast-id database of the National Center for Biotechnology Information (NCBI) (blast.ncbi.nim.nih.gov/Blast.cgi)) to identify them by sequence homology with described yeast taxa.

The sequences of the D1/D2 26S rRNA gene of isolates identified as *Candida albicans* and closely related taxa retrieved from BLASTN search were included in multiple alignments generated using MEGA6 (Tamura *et. al.*, 2013). The Kimura 2-parameter model was used for distance correction, and the Neighbor-Joining method was used for phylogenetic inference. Support for tree branches was evaluated by bootstrap analysis from 1000 heuristic searches.

3.12 Microbial Analysis

3.12.1 Serial dilution of milk samples

A 10 fold serial dilution was done using 3 tubes containing 9mls of sterile physiological saline (PSS). One milliliter of the milk sample to be tested was added to the first tube and mixed thoroughly. Next, 1ml of the diluted tube was again taken into the subsequent tube and mixed thoroughly. The exercise was continued in that order until desired concentrations were reached.

3.12.2 Inoculation of culture media

For the purpose of this study, the following culture media were employed and prepared according to manufacturer's instruction.

a. MacConkey Agar for Coliform counts
b. Nutrient Agar for Total plate counts

Serially diluted milk samples were inoculated onto culture media by surface plating. In this case, from dilution 10^{-2}, 0.1ml was used to inoculate MacConkey while,from dilution 10^{-3}, 0.1ml was used to inoculate Nutrient Agar plates.

Plated culture media were aerobically incubated at 37°C for 24 hours. Colonies on culture media were counted and expressed as colony forming units per milliliter (CFU/ml) of the sample.

3.12.3 Purification of coliform colonies

Individual pinkish colonies on MacConkey agar was picked and subcultured on a freshly prepared MacConkey agar plate and incubated at 37°C for 24 hours to get pure culture of the isolate. Representative colonies were stored on slant of nutrient agar and kept in the refrigerator (4°C) until required for further work(David, 2011).

3.13 Biochemical Tests for Coliforms

Conventional biochemical tests (TSI, SIM, Citrate, Urea and MRVP) were done to preliminarily identify the bacteria isolates before using a commercial kit for

Enterobacteriaceae(Medica-Tec^{TM} Microgen GN-IDA+BKit) to confirm the identities of the isolates according to the manufacturers' instructions.

3.14 Antibiotic Sensitivity Testing

Antibiotic sensitivity was carried out on thecoliform isolates using commercially prepared antibiotics (Oxoid). The antibiotics employed were: Gentamicin, Amoxicillin, Chloramphenicol, Streptomycin, Ciprofloxacin, Tetracycline and Erythromycin.

Colonies of the isolate of interest was inoculatedinto peptone water;the inoculum was standardized to 0.5 McFarland and incubated for 4 hours. A sterile cotton swab was soaked into the peptone water culture and excess fluid expressed from the swab by rolling it on the side of the culture bottle. The swab was used to streak the entire surface of the Mueller-Hinton agar plate. Then antibiotic discs were placed on the surface of the plate 15 mm apart. The plates were then incubated at 37°C for 24 hours.

The zone of inhibition was measured in millimeters (mm) and compared against a reference standard which contains measurement ranges and their equivalent qualitative categories of susceptible/sensitive (CLSI, 2013).

3.15 Administration of Questionnaires

A structured questionnaire was developed and administered through discussion with the Zonal Veterinary Assistants and Livestock Extension Specialists.The questionnaire was in two parts: herd/farm and individual cow information.

3.15.1 Herd information

Herd information was supplied by the owner on questions like: Name of farm, location, cattle population, decrease in milk yield, type of management practice, sanitary condition of farm, disinfection of milking containers, are the cows uddercleaned and towelled dry before milking, type of milking method use and does the farm follow a planned breeding program.

3.15.2 Individual cow information

The cattle handlers/herdsmen supplied information on the age of animal, parity, mastitis status,time of mastitis onset andpresent state of udder.

A total of 300 questionnaires composed in English were distributed and translated to the Fulani herdsmen in Hausa language. Each questionnaire took about 10-15 minutes to be administered.

3.16 Data Analyses

The Statistical Package for Social Science (SPSS) version 20 was used to analyze the data. Each cow-in-milking was a statistical unit. Outcome of mycological examination, sensitivity to antimycotics, coliform examination and sensitivity to antibiotics were compiled in tabular forms. Prevalence was calculated as number of yeasts isolated as the numerator with total number of isolates as denominator.

Data obtained from questionnaires were analyzed to determinethe riskfactors of subclinical mastitis anddistribution of disease in cows of thestudy area. The prevalence (number of

affected cows as the numerator with total number of cows tested as denominator) was calculated. Significance of risk factors onthe prevalence of mastitis in cows was calculatedusing chi-square ($\chi 2$) technique to test the association between affected cows (CMT positive) and risk factors likeage, parity and stage of lactation.

In addition,logistic regression analysis was used to calculate odds ratio (with 95% confidence interval and $P < 0.05$ regarded as significant)to measure the degree of association between risk factorsand the disease in cows.Results of different isolates were compared.

CHAPTER FOUR

4.0 RESULTS

4.1 Diversity of Fungal Isolates in Milk Samples and Antimycotic Sensitivity Testing

Thirty-seven fungal isolates were recovered from 300 milk samples in Kaduna State using API 20 C Aux kit. The fungal species and their frequencies of isolation were: *Candida* spp. (54.1%), *Trichosporon mucoides* (13.5%), *Cryptococcus* spp.(8.1%),*Saccharomyces cerevisiae*(5.4), *Stephanoascus ciferrii*(5.4%), *Rhodotorula mucilaginosa* (5.4%),*Kloeckera* spp. (2.7%),*Kodamaea ohmeri* (2.7%) and*Geotrichum capitatum*(2.7%), (Table 4.1). Two hundred and sixty-three (87.7%) cow milk samples were negative for fungal growth (Tables 4.7 and 4.8).

Twenty(54.1%) of the isolates belonged to the genus*Candida*. The species of *Candida* isolated from cows with mastitis are presented in Table 4.1, and the most frequently isolated species were *C. albicans* (24.3%), *C. famata* (10.8%) and *C. krusei* (10.8%).Allthe 9 *Candida albicans*isolates tested were positive for germ tube and chlamydospore formation.

*In vitro*sensitivity of fungal isolatesshowed *Candida* spp. (85%) sensitive to griseofulvin and amphotericin B. All the isolates of *Cryptococcus* spp., *Stephanoascus* spp. and *Kloeckera* spp. were sensitive to amphotericin B while, 80% of *Trichosporon* spp. was sensitive to amphotericin B. *Kodamea* spp. was resistant to all the antimycotic agents used. In terms of decreasing efficacy of antimycotics on isolates tested in this study, antimycotics can be ranked asfollows: Amphotericin > Griseofulvin > Nystatin > Variconazole > Fluconazole (Table 4.2).

Table 4.1:Identification of fungal isolates from 300 cow milk samplesin Kaduna metropolis, Kaduna State, Nigeriausing API 20 C Aux kit.

S/No	Fungi	Frequency of specie isolation n (%)	Frequency of genus isolation n(%)
1	*Candida albicans*	9 (24.3)	20(54.1)
2	*Candida famata*	4(10.8)	
3	*Candida krusei*	4(10.8)	
4	*Candida boidinii*	1(2.7)	
5	*Candida pelliculosa*	1(2.7)	
6	*Candida lusitaniae*	1(2.7)	
7	*Trichosporon mucoides*	5(13.5)	5(13.5)
8	*Cryptococcus humicola*	1(2.7)	3(8.1)
9	*Cryptococcus laurentii*	1(2.7)	
10	*Cryptococcus albidus*	1(2.7)	
11	*Saccharomyces cerevisiae*	2(5.4)	2(5.4)
12	*Stephanoascus ciferrii*	2(5.4)	2(5.4)
13	*Rhodotorula mucilaginosa*	2(5.4)	2(5.4)
14	*Kloeckera* spp.	1(2.7)	1(2.7)
15	*Kodamaea ohmeri*	1(2.7)	1(2.7)
16	*Geotrichum* capitatum	1(2.7)	1(2.7)
	Total	**37(100)**	**37(100)**

Table 4.2: *In vitro* sensitivity testing of fungal isolates from cow milk in Kaduna metropolis, Kaduna State, Nigeria.

Fungal species isolates	No. of isolates	No (%) SENSITIVE TO EACH ANTIMYCOTIC NYS	VOR	FLU	GRI	AMP
Saccharomyces spp.	2	1(50)	1(50)	1(50)	1(50)	1(50)
Candida spp.	20	7(35)	0(0)	0(0)	17(85)	17(85)
Kodamea spp.	1	0(0)	0(0)	0(0)	0(0)	0(0)
Stephanoascus spp.	2	1(50)	1(50)	0(0)	2(100)	2(100)
Cryptococcus spp.	3	0(0)	0(0)	0(0)	1(33.3)	3(100)
Rhodotorula spp.	2	2(100)	2(100)	1(50)	0(0)	1(50)
Trichosporon spp.	5	1(20)	1(20)	0(0)	2(40)	4(80)
Geotricum spp.	1	1(100)	1(100)	0(0)	0(0)	0(0)
Kloeckera spp.	1	0(0)	0(0)	0(0)	1(100)	1(100)

Key:NYS = Nystatin, VOR = Variconazole, FLU = Fluconazole, GRI = Griseofulvin and AMP = Amphotericin B.

4.2 Phylogenetic Identification of Yeast Isolates from Cow Milk in Kaduna Metropolis, Kaduna State, Nigeria

Plate 4.1 shows that eleven yeast isolates were identified by nested PCR. The bands (600bp) generated by PCR of the ITS-5.8S region were identical for the yeast isolates pertaining to the same species. Sequencing of the domains D1 and D2 of the 26S rRNA gene showed that the isolated yeasts belonged to the same species.Identification analysis revealed three (3)isolates of *Candida albicans*, three (3) isolates of *Pichia kudriavzevii* (also known as *Candida krusei*)andfive (5) isolates of *Saccharomycescerevisiae*.

Phylogenetic reconstruction based on the D1/D2 26S rRNA gene sequences of the 11 isolates grouped them into 3 clusters (Figure 4.1). Clusters 1 and 2 showed that all the isolates (7) identified as *Pichia kudriavzevii* formeda monophylectic group with 93-100% nucleotideidentity among themselvesand were most closely related to two of the isolates identified as *Candida albicans* with a73.3-73.9% nucleotide identity.However, their nucleotideidentity with one of the *C.albicans* strain wasbetween 41.1-41.7% (Figure 4.2). Cluster 3 showed that out of the three *C.albicans* strains identified, two were100%similar but were not as identical as the other *C. albicans* isolatein the same monophylectic group. The D1/D2 26S rRNA gene sequence of strains in clusters 1, 2 and 3 differed from the *Saccharomyces*identified. Although, Saccharomyces was most closely related to *P.kudriavzevii* strains (52.7% nucleotide identity) than to the *C. albicans* strains (43.9%) (Figures 4.1 and 4.2).

Comparison of the isolatesinthis study with those from the GenBank (Table 4.3) showed thatthesequences of the newly identified isolates seem to be different from the available sequences accessed with 19-36% nucleotide identity(Figure 4.2).

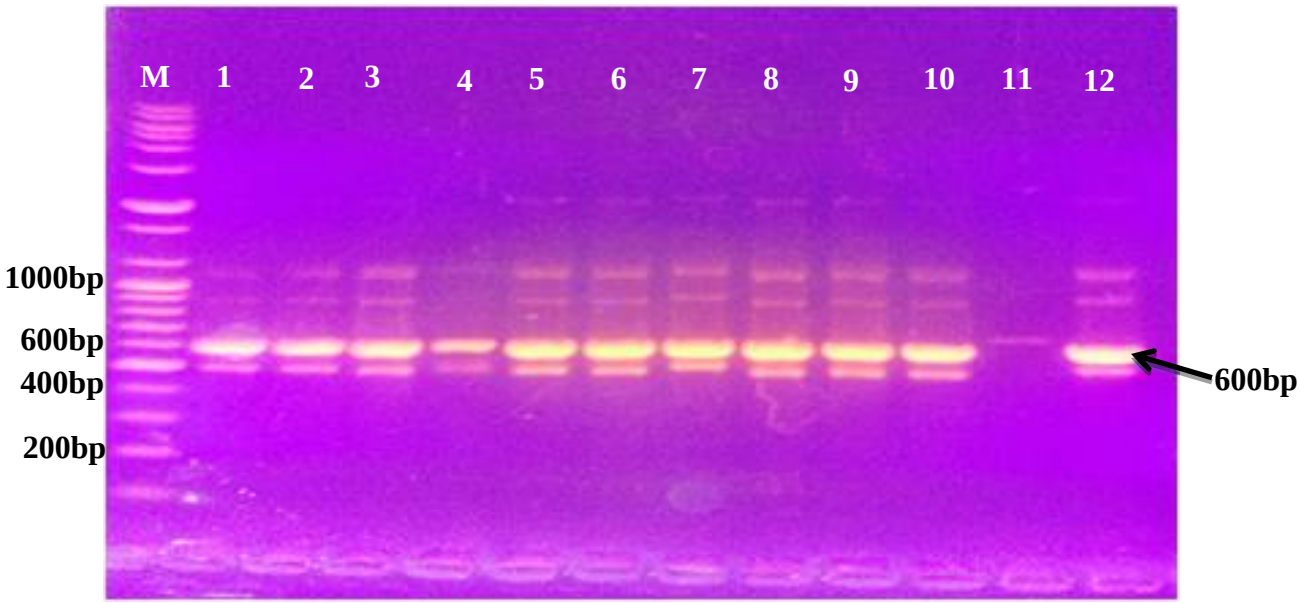

Plate 4.1: **Nested PCR of yeast isolates' DNA in bovine milk showing amplified D1/D2 domains of the 26S rRNA gene.**

Key:

Lane M	=	100bp ladder (Bioneer)
Lane 1	=	B18 *Candida albicans*
Lane 2	=	D35 *Candida albicans*
Lane 3	=	D38 *Pichia kudriavzevii*
Lane 4	=	F54*Pichia kudriavzevii*
Lane 5	=	J101*Pichia kudriavzevii*
Lane 6	=	M142*Saccharomyces cerevisiae*
Lane 7	=	O155 *Saccharomyces cerevisiae*
Lane 8	=	S201 *Saccharomyces cerevisiae*
Lane 9	=	V247*Saccharomyces cerevisiae*
Lane 10	=	Z282 *Saccharomyces cerevisiae*
Lane 11	=	-ve Negative control
Lane 12	=	+ve Positive control (D34/*Candida albicans*)

Table 4.3: GenBank accession numbers of sequences of 26S rRNA genes of fungal isolatesused for phylogenetic analysis

Fungi ID Designations%	Country of origin	Sampling Year	GenBank accession no.
*Candida albicans*95	China	2014	KM013360
*Candida albicans*98	South Africa	2016	KJ534505
*Candida albicans*98	USA	2015	KP780462
Pichia kudriavzevii 97	Iran	2009	GS599266
*Pichia kudriavzevii*98	Italy	2012	JX129895
*Pichia kudriavzevii*99	Brazil	2014	LC015306
*Saccharomyces cerevisiae*91	South Africa	2014	KP070744

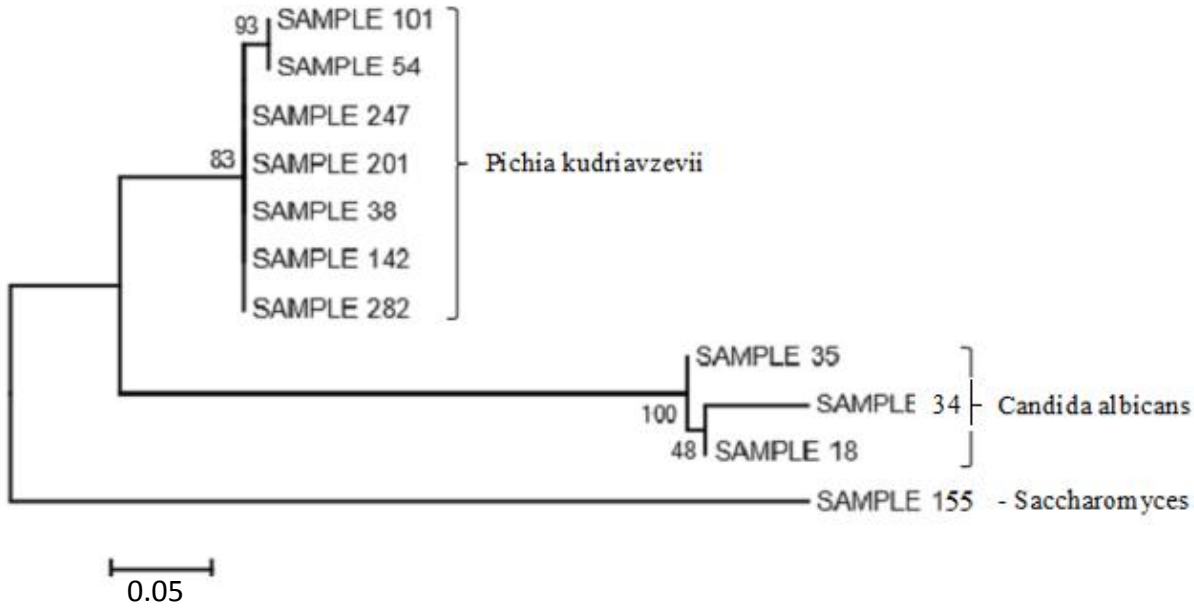

Figure 4.1: **Midpoint neighbor-joining phylogeny tree analysis of the strains of fungi isolates from milk samples in Kaduna metropolis, Kaduna State, Nigeria.**

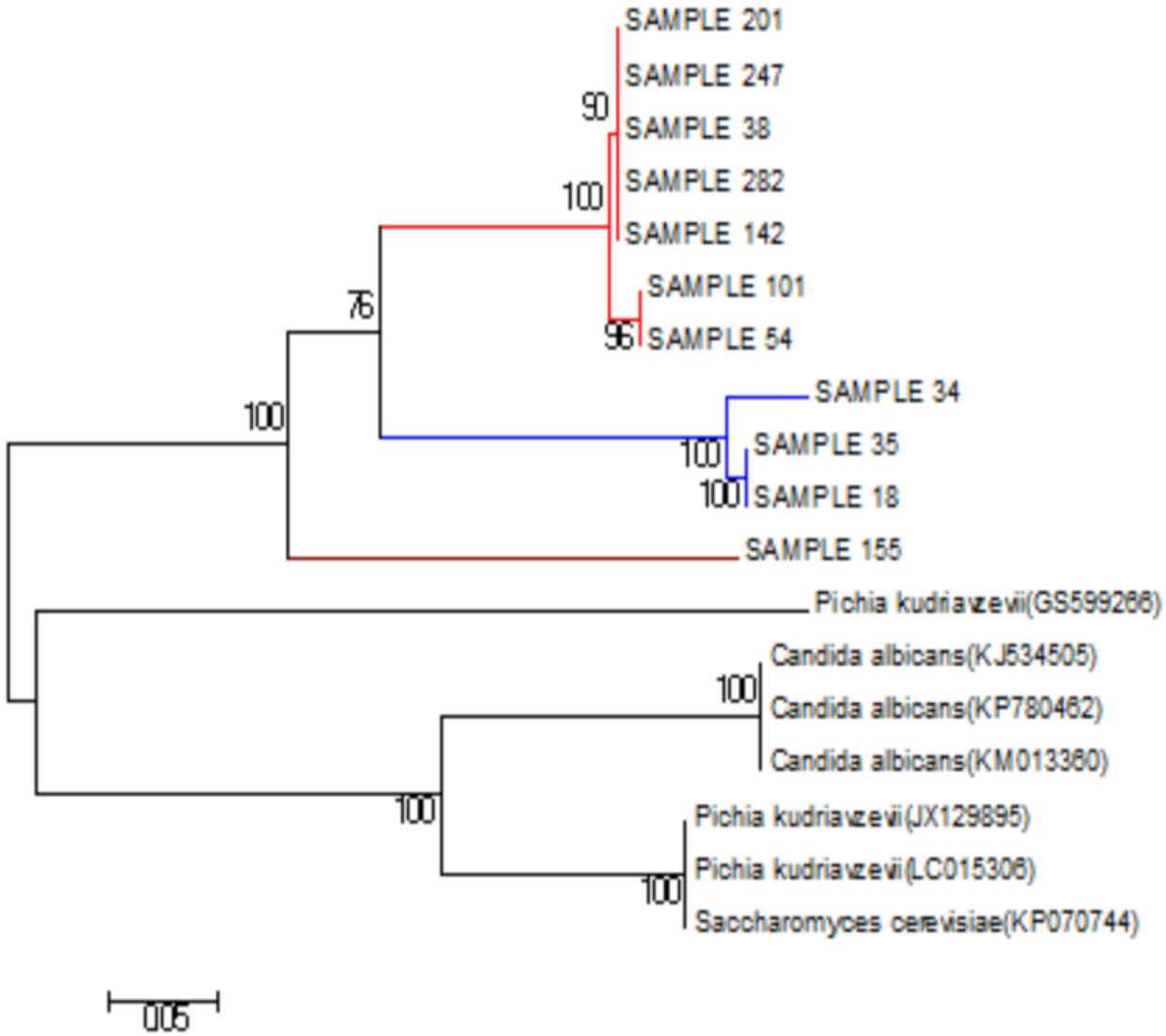

Figure 4.2: **Midpoint neighbor-joining phylogeny tree analysis of the strains of fungi isolates from cow milk samples in Kaduna metropolis, Kaduna State compared with availablesequences of fungi strains from the GenBank.**

4.3 Diversity of Coliform Isolates in Cow Milk Samples and AntibioticSensitivity Testing

Table 4.4, shows the distribution of Coliform bacteria from 300cow milk samples identifiedusing Microgen GN-ID A+B Kit (Medica-Tec^{TM}).Thirty-one milk samples were culturally positive for coliformsrepresenting 10.3% while, 269 (89.7%) milk samples were negative. More than half of the isolates belonged to the genus*Klebsiella* accounting for 51.6%, the others were *Enterobacter* (12.9%), *Citrobacter*(9.7%), *Proteus*(9.7%), *Serratia*(13%) and *Pantoea*(Similar to *Enterobacter*) (3.2%). Two hundred and sixty-nine (89.7%) cow milk samples were negative for coliform isolates (Tables 4.7 and 4.8).

Sixteen cow milk samples were positive for *Klebsiella* spp. (51.6%). The species of *Klebsiella* isolated from cows with mastitis are presented in Table 4.4, and the most frequently isolated ones were *Klebsiella pneumoniae* (35.5%) and *Klebsiella oxytoca* (16.1%).

Table 4.5, shows the *in vitro* sensitivity of bacterial isolates to a panel of 7 antimicrobials. Amoxicillin was found to be the most effective among all antibiotics used in thepresent study, as all the31 (100%) bacterial isolates were sensitive to it. This was closely followed by ciprofloxacin where all but 6(37.5%) bacterial isolates belonging to the genus *Klebsiella* where resistant. Erythromycin was the least sensitive among the antibiotics tested in this study as only one bacterial isolate belonging to the genus *Serratia* was sensitive to it.In terms of decreasing sensitivity of antibiotics on isolates, the antibiotics used in this study can be ranked as follows: amoxicillin > ciprofloxacin >gentamicin> chloramphenicol> streptomycin> tetracycline > erythromycin.

Table 4.4: Identification ofcoliform bacteria isolated from 300 cow milk samples from Kaduna metropolisusing Microgen GN-ID A+B Kit (Medica-Tec^{TM})

S/No	Bacteria isolated	Frequency of specie isolation n(%)	Frequency of genus isolation n(%)
1	*Enterobacter cloacae*	1(3.2)	4(12.9)
2	*Enterobacter aerogenes*	1(3.2)	
3	*Enterobacter gergoviae*	2(6.5)	
4	*Citrobacter freundii*	2(6.5)	3(9.7)
5	*Citrobacter koseri*	1(3.2)	
6	*Klebsiella pneumoniae*	11(35.5)	16(51.6)
7	*Klebsiella oxytoca*	5(16.1)	
8	*Serratia marcescens*	4(13.0)	4(13)
9	*Proteus mirabilis*	3(9.7)	3(9.7)
10	*Pantoea agglomerans*	1(3.2)	1(3.2)
	Total	**31(100)**	**31(100)**

Table 4.5: *In-vitro*sensitivitytesting of bacterial isolatesfrom cow milk in Kaduna metropolis, Kaduna State, Nigeria to seven antibiotics

Bacterial species	No. of isolates	No (%) SENSITIVE TO EACH ANTIBIOTIC C	A	CH	S	T	G	E
Enterobacter spp.	4	4(100)	4(100)	2(50)	2(50)	1(25)	2(50)	0(0)
Citrobacter spp.	3	3(100)	3(100)	1(33.3)	1(33.3)	1(33.3)	2(66.7)	0(0)
Klebsiella spp.	16	10(62.5)	16(100)	10(62.5)	10(62.5)	5(31.3)	10(62.5)	0(0)
Serratia spp.	1	44(100)	4(100)	2(50)	0(0)	0(0)	4(100)	2(50)
Proteus spp.	3	3(100)	3(100)	2(66.7)	0(0)	0(0)	3(100)	0(0)
Pantoea spp.	1	1(100)	1(100)	1(100)	0(0)	0(0)	1(100)	0(0)

Key: C = Ciprofloxacin, A = Amoxicillin, CH = Chloramphenicol, S = Streptomycin, T = Tetracycline, G = Gentamicin and E = Erythromycin

4.4 Prevalence of Mastitis among Sampled Farms

In this study, 26 farms were sampled from four Local Government Areas (LGAs) that make up Kaduna metropolis in Kaduna State namely; 5(19.2%) from Chikun, 8(30.8%) from Igabi, 8(30.8%) from Kaduna north and 5(19.2%) from Kaduna south (Table 4.6). A total of 300 cows in-milking were examined; 89(29.7%) were positive for mastitis at animal level (CMT)among these,32(10.7%) clinical cases of mastitis while 57(19%) were subclinical cases. Of 1,200 quarters examined 12(1%) were blind, leaving 1188 quarters functional. Out of the total quarters examined, 205(17.1%) were affected: 32(2.7%) clinically and 173(14.4%) subclinically.

Laboratory examinations showed, 13(4.3%) milk samples were contaminated with only yeasts, 7(2.3%) milk samples were contaminated with coliforms only, while, 24(8%) milk samples were contaminated with both yeasts and coliforms (Table 4.7). According to LGAs grouping of the dairy farms sampled; Igabi recorded the highest number of milk samples 34(36.2%) positive by CMT and also, the highest number of yeasts contaminated milk samples 14(37.8%).While, Chikun had highest percentage of milk samples contaminated with coliforms 8(25.8%) (Table 4.8).

Regarding age of cows, significant effect ($P < 0.05$) was observed (Table 4.9) on the prevalence of mastitis. There was variation between the age of the animal and mastitis prevalence; the lowest prevalence (24%, 48 of 200) of mastitis wasfound in cows of 3-4 years of age, while the highest (60.6%, 20 of 33) in the animals aged above 5 years (Table 4.10).In relation to parity number, highly significant effect ($p<0.05$) wasfound on the prevalence of mastitis. Theprevalence of mastitis rose with an increase in paritynumber which has been shown very clearly in Tables 4.9 and4.10 where 37.2% (35 of 94) of cows with >3 parity had

mastitis.Stage of lactation was significantly affected ($p<0.05$) and wasfound to be associated with the prevalence of mastitis(Table 4.9) being the highest (45.5%; 30 of 66)during the initial stage of lactation (0 to 5 months)followed in order by more than 10 months as43.3% (39 of 90),(Table 4.10).

There was asignificant difference ($p<0.05$) in the prevalence ofmastitis between management systems (Table 4.9): higher prevalence (34.6%; 85 of 246) of mastitis was found in cows raised under nomadic system as compared to that of sedentary system (31.5%; 17 of 54) (Table 4.10). A significant difference ($p<0.05$) was observed on theprevalence of mastitis between cows kept underdifferent milking hygiene process: higher prevalence ofmastitis in cows with poor milking hygiene (61.5%; 88 of 143) than in cows with good milking hygiene (14%; 22 of 157). Inaddition, a significance difference ($p<0.05$) wasnoted on the prevalence of mastitis between cowswith lesion on the udder/teat skin (38.7%; 24 of 62) and cows without this factor (29.8%; 71 of 238),(Tables4.9 and 4.10).

Table 4.6: Prevalence of bovine mastitis in four selected LGAs sampled in Kaduna metropolis, Kaduna State, Nigeria

Location	No. sampled	No. (%) positive	$\chi 2$	df	p-value
No. of farms					
Chikun	5	2(40%)	0.680	3	0.954
Igabi	8	2(25%)			
Kaduna north	8	2(25%)			
Kaduna south	5	2(40%)			
No. of animals					
Chikun	65	12(18.5%)	5.893	3	0.117
Igabi	94	34(36.2%)			
Kaduna north	90	27(30%)			
Kaduna south	5116	(31.4%)			

Table 4.7: Farm distribution in LGAs of CMT positive cow milk samples showing number of single infection and mixed infections by yeasts and coliforms

S/no	Farms In LGA	No. of samples examined	No of sample +ve for CMT	No of sample +ve for yeast	No of sample +ve for coliform
Kaduna north					
1	A1	95	1	0	
2	A2	11	3	1	0
3	A3	10	3	2	2
4	A4	11	1	1	0
5	A5	12	4	1	1
6	A6	10	0	0	0
7	A7	14	10	3	1
8	A8	13	1	2	0
Igabi					
9	B1	12	5	3	3
10	B2	11	4	0	2
11	B3	5	0	0	0
12	B4	16	2	2	0
13	B5	15	3	3	1
14	B6	12	12	6	5
15	B7	13	1	1	1
16	B8	10	7	1	1
Chikun					
17	C1	8	2	2	3
18	C2	14	0	0	0
19	C3	11	2	0	1
20	C4	18	3	2	2
21	C5	14	5	2	2
Kaduna south					
22	D1	9	1	0	1
23	D2	14	7	3	3
24	D3	9	0	0	0
25	D4	11	2	0	2
26	D5	8	1	0	
Total		**300**	**89(29.7)**	**37(12.3)**	**31(10.3)**

Table 4.8: Selected four LGAsshowing the summary of CMT positives, yeast positives and coliform positives

S/No	Name of LGA	No of animals sampled	No of animals CMT +ve (%)	No of animals yeast +ve (%)	No of animal coliform +ve (%)
1	Kaduna north	99	27(27.3)	11(29.7)	4(12.9)
2	Igabi	94	34(36.2)	14(37.8)	13(14.9)
3	Chikun	65	12(18.5)	6(16.2)	8(25.8)
4	Kaduna south	51	16(31.4)	4(10.8)	6(19.4)

Table 4.9: Association of risk factors of mastitis with occurrence of mastitis in cows in Kaduna metropolis, Kaduna State, Nigeria using logistic regression analysis

Risk factor	P value	Crude odds ratio (95%CI)	Adjusted odds ratio (95%CI)
Age (years)	0.03	4.7(2.4–10.6)	3.3(1.6–7.2)
Parity	<0.01	3.3(1.7–7.8)	2.1(1.8–5.8)
Stage of lactation	0.03	9.1(4.2–19.8)	4.0(1.7–9.5)
Management system	0.04	10.1(3.9–21.2)	4.0(1.9–8.4)
Milking hygiene	<0.01	8.7(4.4–17.3)	3.4(1.6–10.2)
Lesion on udder/teat	<0.01	21.8(7.9–68.5)	10.7(3.8–36.5)

Table 4.10: Different determinants influencing the prevalence of bovine mastitis in Kaduna metropolis, Kaduna State, Nigeria

Parameter	No. of cows examined	No. of affected cows(CMT+ve)	Prevalence (%)
Age (years)			
<2	67	21	31.3
3–4	200	48	24.0
>5	33	20	60.6
Parity			
1	83	21	25.3
2	123	33	26.8
>3	94	35	37.2
Stage of lactation (months)			
0–5	66	30	45.5
6–10	144	16	11.1
>10	90	39	43.3
Management system			
Sedentary system	54	17	31.5
Nomadic system	246	85	34.6
Milking hygiene			
Good	157	22	14.0
Poor	143	88	61.5
Lesion on udder/teat			
Yes	62	24	38.7
No	238	71	29.8

4.5 Farm Practice and Diseases Common among Farmers

The survey showed that 25(96.2%) of the farms practiced hand milking method and milked cows once daily while, the only (3.8%) farm that milked with machine milked cows twice daily. Among the 26 farms studied, 21(80.8%)allowed calves to suckle for a few minutes before milking commenced, 4(15.4%) washed the udder with only water before milkingwhile,1(3.8%) washed with soap and water before milking cows. Twenty (77%) farms usedplastic buckets for milk collection while, 5(19.2%) used the traditional calabash and 1(3.8%) used stainless steel bucket for milk collection. Over 96% of the farms washed milk collection containers with water only and 24(92.3%) farms reported a decrease in milk yield (Table 4.11).

Allfarmers in this study have heard of mastitis and among these 20(77%) farmshave had casesofmastitisbefore while, 6(33%) have not. Twenty-one (80.8%) farms use veterinary services only while 5(19.2%) used both veterinary services and traditional methods to treat their sick cows. All the 26farmers consumed milk from their cows and 21(80.8%) said they boiled milk before drinking while, 5(19.2%) drank raw milk. All the herdsmen said they had been sick in the past after consuming cow milk. Eleven (42.5%) of the herdsmen reported having suffered from sore-throat,6(23%) from campylobacteriosis while,9(34.5%) had suffered fromthrush,tuberculosis ortyphoidaccording to hospital diagnosis (Table 4.12).

Table 4.11: Milking practices on farms

Variable	No	Frequency (%)
Milking methods		
Hand	25	96.2
Machine	1	3.8
No of milking times/day		
Once	25	96.2
Twice	1	3.8
Udderpreparation before milking		
Calves suck before milking	21	80.8
Wash with soap andwater	1	3.8
Wash with water only	4	15.4
Type of milking container		
Plastic	20	77.0
Calabash	5	19.2
Stainless steel	1	3.8
Cleaning of milking container		
Wash with water only	25	96.2
Wash with water and soap	1	3.8
Is there decrease in milk yield?		
Yes	24	92.3
No	2	7.7

Table 4.12: Common diseases affectingfarmers

Variable	No	Frequency(%)
Have you heard of mastitis?		
Yes	26100	
No	0	0
Have your cows had mastitis before?		
Yes20	77	
No6	33	
What do you dowhen your cows are sick?		
Report to Veterinarian	21	80.8
Use veterinarian and traditional methods	5	19.2
Do you consume milk from your cows?		
Yes	26	100
No0	0	
What do you do before you drink milk?		
Boil/Pasteurize	21	80.8
Don’t boil/pasteurize	519.2	
Have you been sick after consuming milk?		
Yes	26	100
No0	0	
If yes, what was it?		
Sore-throat	11	42.5
Campylobacteriosis	6	23.0
Thrush	3	11.5
Tuberculosis	3	11.5
Typhoid	3	11.5

CHAPTER FIVE

5.0 DISCUSSION

5.1 Discussion

This study showed that 37 yeast isolates were identified from 300 cow milk samples using API 20 C Aux as:*Candida* spp., *Trichosporon mucoides, Cryptococcus* spp., *Saccharomyces cerevisiae, Stephanoascus ciferrii, Rhodotorula mucilaginosa, Kloeckera* spp., *Kodamaea ohmeri* and *Geotrichum capitatum*which agrees with some worksthatreported the most common aetiological agents of mycoticmastitis as: *Candida*,*Cryptococcus*, *Rhodotorula*, *Trichosporon, Torulopsis,*and *Geotrichum* (Krukowski *et al*., 2000; Spanamberg *et al*., 2008). In tropicalcountries, more species of yeast-like fungi, as well as mildew fungi (*Aspergillus, Penicillum, Epicoccum, Phoma,* and *Alternaria*) may be found in the infected mammary secretions (Władysław *et al*., 2010).

The percentage frequency of isolation of fungiin this study was 12.3%, with 6.7% of theisolates belonging to the genus*Candida*. The rate of *Candida* spp. isolation (6.7%) was higher than the rate of isolation of other yeasts and yeast-like organisms (5.6%).The findings in this studyagrees with the reports by other authors, who demonstrated that *Candida* yeasts were the most commonly isolated aetiological agents of mycotic mastitis in cows and the most frequent and abundant species included *Candida albicans*, *Candida krusei* (*Pichia kudriavzevii*)and *Candida kefyr* (Santos and Marin, 2005; Władysław *et al*., 2010).The percentage frequency of isolation of fungi varies considerably, withas high as 25.2% reported in Egypt (Abd El-Razik *et al*., 2011),17.0% in Tanzania (Kivaria and Noordhuizen, 2007), 7.1% in Poland (Władysław *et al*., 2010), 8.9% in Brazil (Santos and Marin, 2005)and 10.2% from camels in Kano state, Nigeria (Kalla *et al*., 2008).These results show that miscellaneous bovine-mastitis pathogens such as yeasts and other fungal species may be an increasing

problem probably due to unhygienic udder preparation, contamination, unsanitary intramammary infusions practices, and indiscriminate use of antibiotics, particularly tetracyclines (Radostits *et al*., 2007). The relatively high prevalence (12.3%) of yeast in bovine mastitis in this study found indairy herds could significantly reduce milk production with resultant economic losses.

Candida albicans isolates have the ability to produce different varieties of virulence factors that help it in adherence, pathogenesis and inducing the disease process in mammary glands. All the *C. albicans*(9) isolates in this study were able to survive in high temperature and produced chlamydospores and germ tubes (hyphal formation) which are considered as confirmatory for identification of *C. albicans* (Zaini *et al*., 2006; Yang, 2008). The source of these pathogenic yeasts in milk from this study was not clear. However,the milk samples may have been contaminated by infected persons during milking process or from the environment, as the organism is ubiquitous. Before now, food products have generally been considered as insignificant vehicles for transmission of pathogenic yeasts (Fleet, 2007). However, Todd, (2008) reported cases where yeasts were suspected of causing food poisoning and Taylor, (2010) also described the possibility of yeasts being connected with allergic reactions of consumers to food. The present study also, shows that milk could act as a transmission vehicle of the pathogenic yeast *C. albicans*.

Four (10.8%) yeast isolates were identified as*Pichia kudriavzevii*(*Candida krusei*) using the API 20 C Aux,while PCR and sequencing identified7isolates as *P. kudriavzevii*.This yeast is ubiquitous in the environment and can be found in soil, fruits and various fermented beverages. So far, *P. kudriavzeii* is mainly associated with food spoilage, causing surface biofilms in low pH products (Kurtzman *et al*., 2011). But, it is important to note that, many

clinical cases have shown that*P. kudriavzevii* is the 5th most common cause of candidemia in immunocompromised patients (such as AIDS patients) (Kurtzman *et al*., 2011) and is therefore a species of clinical importance.

Cryptococcus species represented 8.1% of the isolates in this study.However, *C. neoformans*, which is considered the most dangerous species, was not identified. But *C. laurentii* seen here have been reported by other authors in cases of mastitis and in tanks used for milk storage (Spanamberg *et al*., 2008). Although there are numerous reports of *Cryptococcus* spp. causing clinical mastitis, only 3 isolates of this genus were identified during this study. *Cryptococcus* spp. has been reported as causing a rather severe clinical mastitis (Akange *et al*., 2013) but since this study was concerned primarily with subclinical infection, fewer *Cryptococcus* isolates were isolated. The cultural methods used may also have been more effective in isolation of *Candida* species.

Among the yeast species found in milk, it is important to highlight the presence of *Kodamaea ohmeri* (*Pichia ohmeri*). This has only been previously found in Brazilian artisanal cheese (Borrelli *et al*., 2006), in cucumber salts (brines) used in the food industry for the fermentation of pickled foods, tree barks and fruits (Deak and Beuchat, 2006), from marine fish in Japan (Li *et al*., 2008) and most recently fromswimming pools (Chakrabarti, 2014). In the past *Kodamaea ohmeri* was considered a contaminant, but it is presently being recognizedas an emerging opportunistic pathogen which can cause infection in patients with special underlying conditions including prematurity, immunosuppression, prolonged hospitalizations, prosthetic valves, peritoneal catheters or other medical devices (Ezeanolue and Piggott, 2007).

The presence of yeasts and yeast-like fungi may trigger alterations in the milk and dairy products due to the release of extracellular enzymes such as lipases and proteinases, which affect the quality and organoleptic characteristics influencing the shelf-life of the product. The diversity of yeasts and yeast-like fungi found in the present study might have been influenced by the type of management system employed in the dairy farms included in this study. Although it is not expected that these microorganisms can survive thermal treatment, milk maybe a carrier for a great diversity of agents that could be harmful to public health (Krukowski *et al*., 2006; Kivariaand Noordhuizen, 2007).

Recent data indicates that, molecular techniques based on PCR have been used as a tool for diagnosis of several fungal species (Bautista-Munoz *et al.*, 2003;Senses-Ergul *et al*., 2006; El-Sharoud *et al*., 2009 and Noumi *et al*., 2009). In the present study, the PCR product of D1/D2 region of all isolatesindicated the presence of 1 distinctive band (600bp).The % identity from blast showed that all theisolates identified as *Pichia kudriavzevii* (also known as*Candida krusei*) were between 97-99% identical to *Pichia*strain; the 3 *Candida*isolates showed 95-98% identity with*C. albicans*, while the only *Sacchromyces cerevisiae*isolatewas 91% identical with *Sacchromyces* strain from the blast.

Phylogenetic reconstruction based on the D1/D2 26SrRNA gene sequences of the 11 isolates grouped them in3 clusters. Clusters 1 and 2 showed that all theisolates (7) identified as *P. kudriavzevii* formed amonophylectic group with 93-100% nucleotide identityamong themselves and were most closely related to twoof the isolates identified as *C. albicans* with a 73.3-73.9%nucleotide identity. However, their nucleotide identity with the third*C. albicans* strain was between 41.1-41.7%.Cluster 3 showed that out of the three *C. albicans* strainsidentified, two were 100% similar but were not asidentical as the third*C. albicans*

isolate in the samemonophylectic group. The D1/D2 26S rRNA genesequence of strains in clusters 1, 2 and 3 differed fromthe *Saccharomyces* identified. *Saccharomyces*was most closely related to *P. kudriavzevii* strains (52.7%nucleotide identity) than to the *C. albicans* strains(43.9%). Comparison of the isolates in this study withthose from the GenBank showed thatthey were different as, the sequences ofthe newly identified isolates were related to those from the GenBank with 19-36%nucleotide identity.

The heterogeneity in the D1/D2 26S rRNA gene of *C. albicans* isolates as shown in this study was not expected, although the sequences of 2 isolates were 100% identical.This is consistent with previous studies on the heterogeneity of the 26S rDNA sequence in other yeast species, such as *C. dublinensis, P. guilliermondii* and *C. lusitaniae*which revealed very few nucleotide substitutions among strains pertaining to the same species (El-Sharoud *et al*., 2009).

Although antimycotic drugs have been used for treatment of yeast mastitis, there is no clear evidence of the effectiveness of this therapy (Crawshaw *et al*., 2005; Krukowski *et al*., 2006).But in this study, 29 (78.4%) of the fungi isolates were sensitive to amphotericin B while, 24 (64.9%) were sensitive to griseofulvin and 13 (35.1%) to nystatin (Table 4.2). In terms of decreasing efficacy of antimycotics on isolates tested in this study, antimycotics can be ranked as follows: Amphotericin B> Griseofulvin > Nystatin > Variconazole > Fluconazole.

The findings of this study highlight the importance of coliform mastitis in lactating cows under the peri-urban condition of Kaduna metropolis, Kaduna State, Nigeria, as 10.3% of the milk samples were positive for coliforms and were in close agreementwith the report of Abdurahman (2006) in eastern Ethiopia and Kalla *et al*.(2008) in Kano State, Nigeria.

However, higher prevalence rates ofcoliform mastitis were reported in India (Sena *et al*., 2000), Southwestern Ethiopia (Woubit *et al*., 2001)and Jordan (Hawari and Hassawi,2008). The highest prevalence of coliform mastitis reported so far in Africawere documented by Junaidu *et al*. (2011) who reported a 52% rate for Sokoto State, Nigeria and Karimurbo *et al*.(2005) who reported 66% in Tanzania. The differences in sample size, environmental conditions, breed and management practices are possibly the major causes for these differences.

The commonly isolated genera of coliform bacteriain this study were*Klebsiella*, *Pantoea*(Similar to *Enterobacter*), *Enterobacter*, *Serratia*, *Citrobacter* and *Proteus*. This is in close agreement with Ameh *et al*., (1993), Matofari *et al*. (2003), Abdurrahman (2006), Kalla *et al*. (2008), Giannino *et al*. (2009), Abera *et al*. (2010) and Garedew *et al*. (2012) who reported*Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter*, *Serratia* and *Proteus* as major mastitogens. Although *E. coli*was not isolated from this study, the other coliform bacteria are generally found in high concentrations in organic matter, such as beddings and manure (environment). Therefore from an epidemiologic standpoint, the primary source of infection for most pathogens in this study was environmental. *Klebsiella* spp. accounted for 51.6% of the isolates in this study; Podder *et al*. (2014) asserted that *Klebsiella pneumoniae* is well adapted to survive in the udder and usually establishes a mild subclinical infection of long duration, from which it is shed in milk, facilitating transmission to healthy animals, mainly during milking procedures. This might be due to the fact that there are no established mastitis control practices that are employed by the farmers, but instead mastitis control relies heavily on drug use. Moreover, the unhygienic housing and milking practices observed among the dairy herds increased both exposure and infection pressure to

cows, with the subsequent high infection levels. The present study found a high prevalence of coliform mastitis and, *Klebsiella* spp. was the dominant coliform isolates in the study area.

Klebsiella spp, *Enterobacter* spp, *Citrobacter* spp. and *Serratia* spp. are usually the coliform bacteria that are most frequently isolated in connection with nosocomial infections (Radostits *et al.*, 2007; Podder *et al*., 2014). Also, of importance is the fact that all species and all serotypes of *Klebsiella*, all species of *Proteus*, *Citrobacter*, *Enterobacter*, *Pantoea* and *Serratia* are listed by the United States Public Health Service, Department of Health and Human Services on bioterrorism list of dangerous biological agents that have the potential to pose a severe threat to public health and safety, to animal health, to plant health, or to animal and plant products (Bynum, 2011). All of these organisms were isolated from cow milk samples in this study and are therefore of public health importance.

Antibacterial susceptibility testingof bacterialisolatesshowed that allthe isolates ofcoliforms were sensitive to amoxicillin.This was followed in decreasing susceptibilities to ciprofloxacin, gentamicin, chloramphenicol, streptomycin, tetracycline and erythromycin. The patterns of resistance exhibited in the present study may be due to prolonged and indiscriminate usage and prescription of particular antibiotics which often leads to resistance development by bacterial agents that infect animals (Kwaga, 2012; Sharma, 2014).

Eighty-nine (29.7%) cow milk samples were CMT positive; of these 13(4.3%) of the milk samples were contaminated with yeasts only, 7(2.3%) contaminated with only coliforms, while, 24(8%) were contaminated with both yeasts and coliforms.

Questionnaire data analysis showed more than 60% of cows aged above 5 years (20 of 33 cows) were affected with mastitis in this study. Age-related results observed in the present study were inconsonance with Sena *et al.* (2001) who reported that animals aged more than 5 years were more commonly affected by mastitis.After the age of 7 years, immunity of the animals is affected,making them vulnerable to diseases. Also in this study, animals with parity number >3 suffered the most from mastitis (37.2%; 35 of 94 cows),which is in agreement the with findings ofAbdurahman (2006). Radostits *et al*. (2005) stated that high yielding animals aremore susceptible to mastitis than low-yielding ones.

In this study, it was observed that cows with lesions on their teats and/or udder (38.7%; 24 of 62 cows) had a 10.7 times more chances of developingmastitis than cows that had no teat lesions (29.8%; 71 of 238 cows). This is similar to the findings of Mulei (1999), who foundin the Kiambu district of Kenya that mammary glandquarters with teat lesions were 7.2 times more likely to havemicroorganisms isolated from them than those withoutany teat lesions.

This study also showed that cows managed under the nomadic system (34.6%; 85 of 246 cows) were 4 times more likely to develop mastitis than cows in the sedentary system (31.5%; 17 of 54 cows). Also, animals with poor hygiene ofmilking process had a 3.4 times chance of developing mastitis. The nomadic system of cattle rearing and poorhygiene of milking process were also identified as riskfactors for occurrence of coliformmastitis in another study inEthiopia (Abdurahman,2006). This might be due to absenceof udder washing, milking cows that had been suckled by calves without washing the udder/teats, milking of cows bymilkerswho had cuts and chaps on their hands, andthe use of common udder cloths, which could be fomites forspread especially for contagious mastitis.

In this study, 21(80.8%) of farmers consumed boiled or pasteurized milk while 5(19.2%) of the farmers consumed raw milk. Although, milk consumption in Africa is fairly low compared to the rest of the world, in tribes where milk consumption is popular, such as the Fulani and Maasai, milk is typically consumed unpasteurized (Mulei, 1999; Ocholi *et al*., 2004).Those favouring the consumption of raw milk believe that raw milk and associated products are healthier and taste better but, under such conditions, public health hazards definitely exist as seen from the type of diseases farmers suffered from; sore throat (42.5%), campylobacteriosis (23%), thrush (11.5%), tuberculosis (11.5%) and typhoid (11.5%).Agencies such as the Centers for Disease Control and Prevention (CDC),Food and Drug Administration (FDA) and other regulatory agencies around the world note that pathogens from raw milk are the causativeagents of tuberculosis, diphtheria, typhoid, and streptococcal infections (FDA, 2009). Similarly, a recent review authored by the Belgian Federal Agency for the Safety of the Food Chain and experts from Belgian universities and institutions concluded that "raw milk poses a realistic health threat due to a possible contamination with human pathogens. It is therefore strongly recommended that milk should be heated before consumption. With the exception of an altered organoleptic (flavour) profile, heating (particularly ultra-high temperature and similar treatments) will not substantially change the nutritional value of raw milk or other benefits associated with raw milk consumption" **(**Claeys*et al*., 2013).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. This study has documented the involvement of yeast (12.3%) and coliform (10.3%) organisms in bovine mastitis in Kaduna metropolis, Kaduna State, Nigeria.

2. The study has shown that all coliform isolates were sensitive to amoxicillin while the yeast organisms were most sensitive to amphotericin B.

3. All *Candida albicans*(9) isolates were considered to be potentially pathogenic, because they survived in high temperature and produced germ tubes in human serum and chlamydospores in corn meal tween 80 Agar.

4. PCR and sequencing of the domains D1 and D2 of the 26S rRNA gene identified eleven yeasts as: *Candida albicans*(3), *Saccharomyces cerevisiae*(1) and *Pichia Kudriavzevii*(*C. krusei*) (7). Phylogenetic reconstruction based on the gene sequences of the 11 isolates showed heterogeneity in *C. albicans*. Also sequences of the newly identified isolates seem to be different from those from the GenBank.

5. The questionnaire survey of thedairy farms showed that the rate of mastitis increased with number of parity. Cows managed under the nomadic system and cows with udder/teat lesions suffered from mastitis the most. Also, all respondents had knowledge of mastitis while 80.8% of them boiledtheir milk before consumption.

6.2 Recommendations

There are no established mastitis control practices that are employed by the farmers despite the potential economic and public health hazard posed by it. Using the data obtained in this study, it is recommended that:

1. Farmers practice good hygiene in milking process, milk clinically infected cows last, cull chronic mastitis cases, treat clinically infected cows and administer dry period therapy. If these are routinely done, the prevalence of mycotic and coliform mastitis in Kaduna State, Nigeria will decrease.

2. The veterinary laboratories should culture, isolate and identify the mastitis microorganisms that are in existence in the country and develop its own classification of mastitis types.

3. Veterinariansshould implement *in-vitro* susceptibility testing prior to the use of antibiotics or antimycotics for treatment of intramammary infections in cows. Also, efforts must be made to encouragedairy farmers to apply safe substitutes such as probiotics and bioactive natural compounds for prophylactic and therapeutic use.

4. Further studies with a larger sample size are necessary to give population representative findings covering the whole country.

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APPENDIX 1

FACULTY OF VETERINARY MEDICINE DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE
AHMADU BELLO UNIVERSITY ZARIA

FARM QUESTIONNAIRE ONPREVALENCE AND RISK FACTORS OF BOVINE MASTITIS DUE TO YEASTS IN KADUNA STATE, NIGERIA

Dear Respondent,

I am Dr. E.U. Mbuk from Faculty of Veterinary Medicine, Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria. I am presently doing my Ph.D. in the above Department as stated in the underlined topic. I need your assistance in filling the questionnaire below as part of the requirement in fulfillment of the Ph.D. Programme. All information given will be treated with utmost confidentiality and strictly for research purposes only.

Thank you.

THE QUESTIONNAIRE

SECTION A: BIODEMOGRAPHIC INFORMATION

Name of LGA:

Town:

Name of farm:

Age of owner:

Date:

SECTION B: ANIMAL INFORMATION

Type of Husbandry practiced:

No. of animals:

No. of female/cows

No. of lactating cows

SECTION C: FARM INFORMATION

1. Type of housing:

   No housing [ ], A shade [ ], Well built up modern dairy house [ ]

2. Type of management practice

   Intensive [ ], Extensive [ ], Semi-intensive [ ]

3. Type of feed

   Legume, concentrate and salt lick [ ], Legume and hay [ ], Hay only [ ]

4. Source of water supply

   Borehole [ ], Pipe borne [ ], Pond [ ], None [ ]

5. Type of hygiene practices on the farm

   Clean paddocks regularly [ ], disinfect and clean the environment [ ], None [ ]

SECTION D: MILKING METHODS

1. What milking methods do you use on the farm?

   Machine [ ], Hand [ ]

2. What do you do to the udder before milking?

   Wash with soapand water [ ], Wash with water only [ ],

   None [ ]

3. How many times do you milk the animals in a day?

   Twice [ ], Once [ ]

4. Have you experience a decrease in the volume of milk produced by your animals?

   Yes [ ], No [ ]

5. What type of container do you use for milking?

   Plastic bucket [ ], Stainless steel bucket [ ], Calabash [ ]

6. How do you clean the milking containers?

   Wash with soap and water [ ], Wash with water only [ ], None [ ]

SECTION E: HEALTH MATTERS

1. What diseases do your animals come down with?

2. Have you heard of mastitis?
   Yes [ ],No [ ]

3. Do your animals have mastitis?
   Yes [ ], No [ ]

4. If yes, what do you do when you observe it?
   Report to a veterinarian[ ], Treat with herbs[ ], Cull/sell-off the animal[ ]
   Leave them alone [ ]

5. Do you consume milk from your cows?
   Yes [ ], No [ ]

6. If yes, what do you do to it before drinking?
   Boil [ ], Pasteurize [ ], None of the above [ ]

7. Have you ever been sick?
   Yes [ ], No [ ]

8. If yes, what was it?
   Tuberculosis [ ],Brucellosis [ ], Campylobacteriosis [ ], Diarrhoea [ ]
   Thrush [ ], Typhoid [ ], Q-fever [ ], Cryptococcosis [ ], Sorethroat [ ]

APPENDIX 2

INDIVIDUAL COW QUESTIONNAIRE

Name of LGA:

Town:

Name of farm:

Age of owner:

Date of collection:

Age [ ]

Parity [ ]

Has it had mastitis before? Yes [ ] No [ ]

Time of onset of mastitis from parturition

What treatment did you give? Infusion [ ], Systemic [ ]

Name of drug(s) used

When was treatment given?

What is the present state of udder?

Use of CMT and Strip cup test to determine the characteristic of milk:

1. Colour [ ]
2. Presence of flakes [ ]
3. Consistency [ ]
4. Presence of clots [ ]
5. Odour [ ]

APPENDIX 3

i. Traditional restraint method of cow for milking

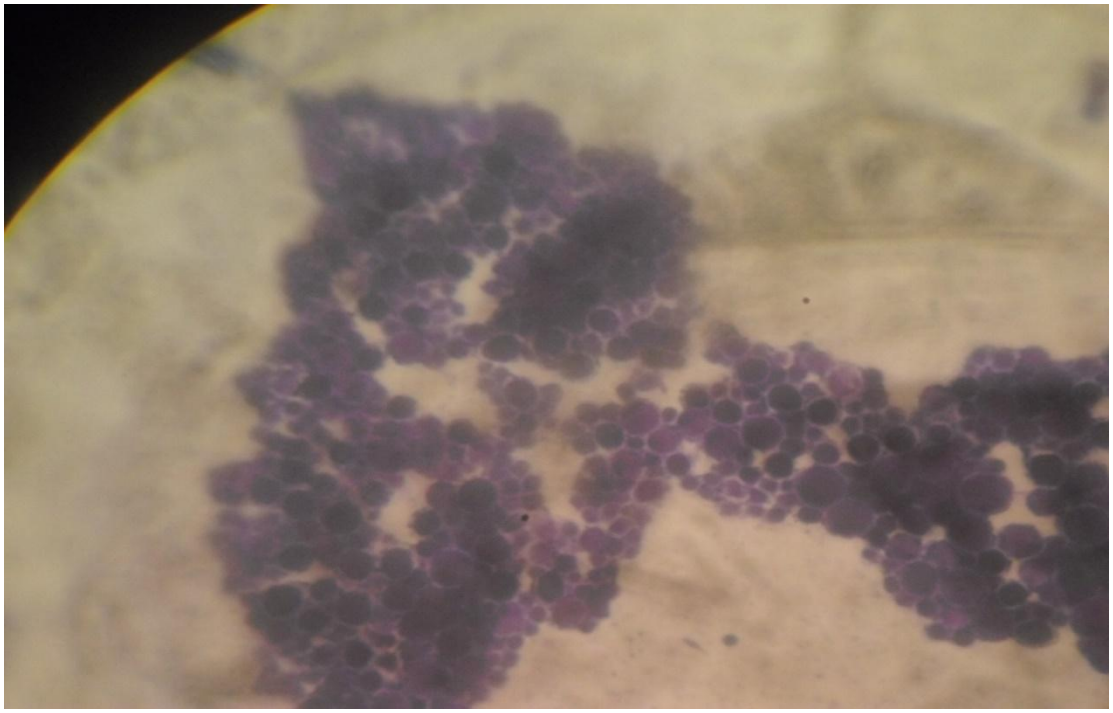
ii. Collection of milk sample for CMT

iii. Paddle showing CMT positive

iv. CMT Sceening on field and collection of pooled milk (10mls) for laboratory investigation

v. Yeast colonies grown on SDA to enumerate fungal counts

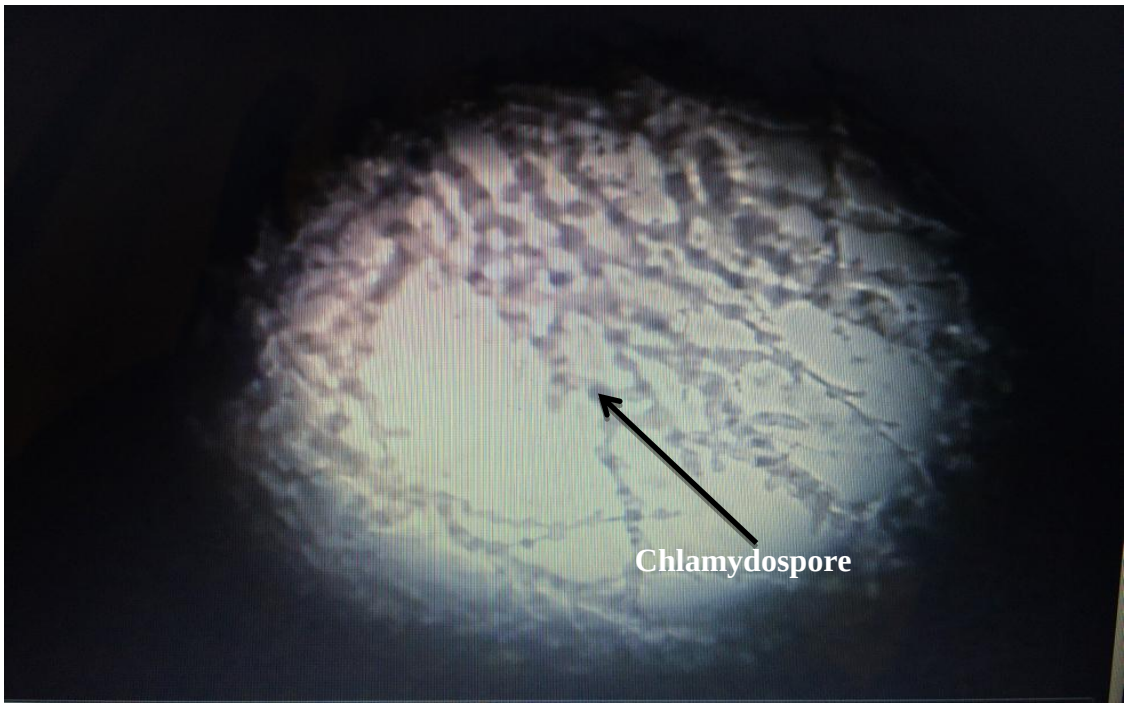

vi. Mold from cow milk grown on SDA



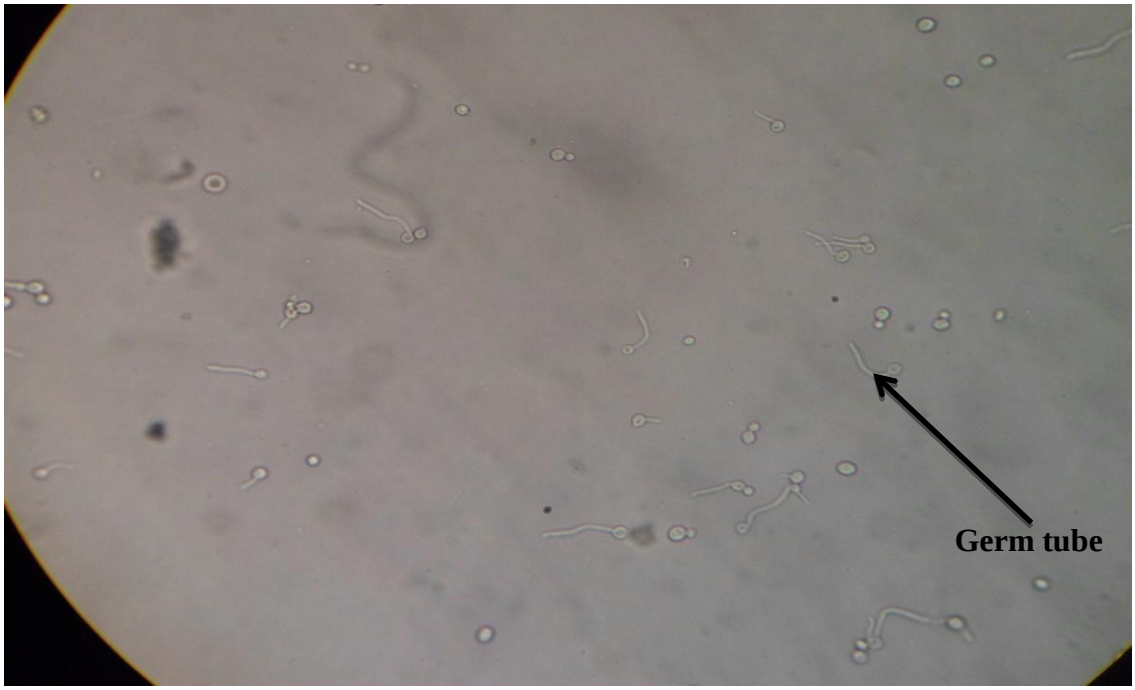
vii. Stained colonies with Lactophenol cotton blue

viii. Yeasts from cow milk grown on SDA containing gentamycin

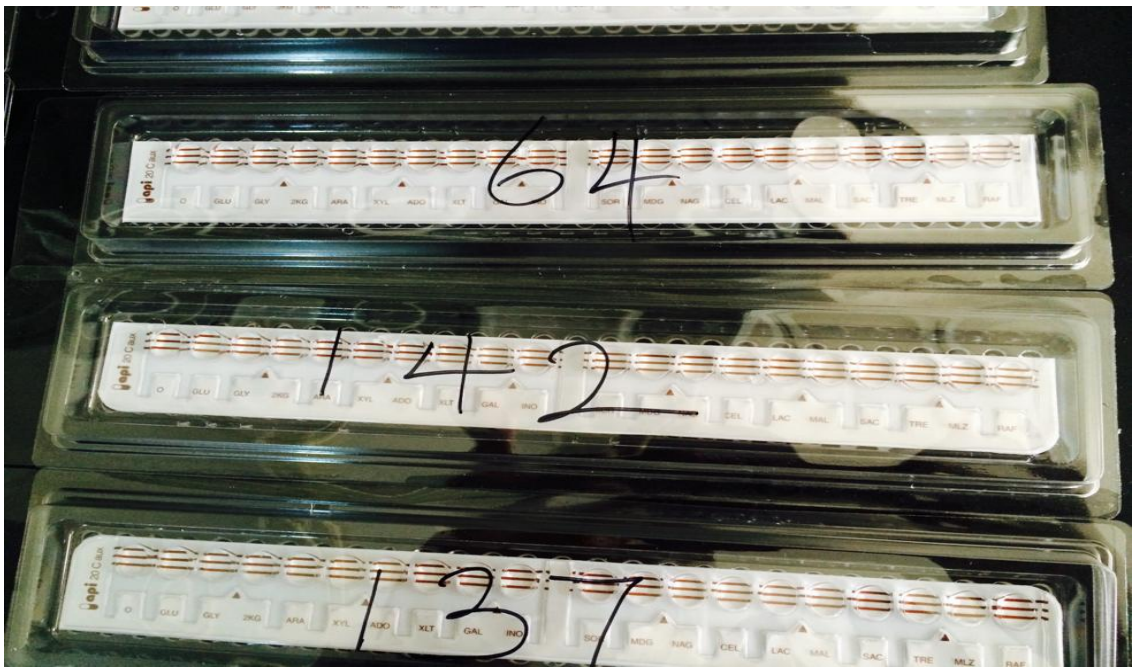
ix. Colonies of yeast stored on SDA slant



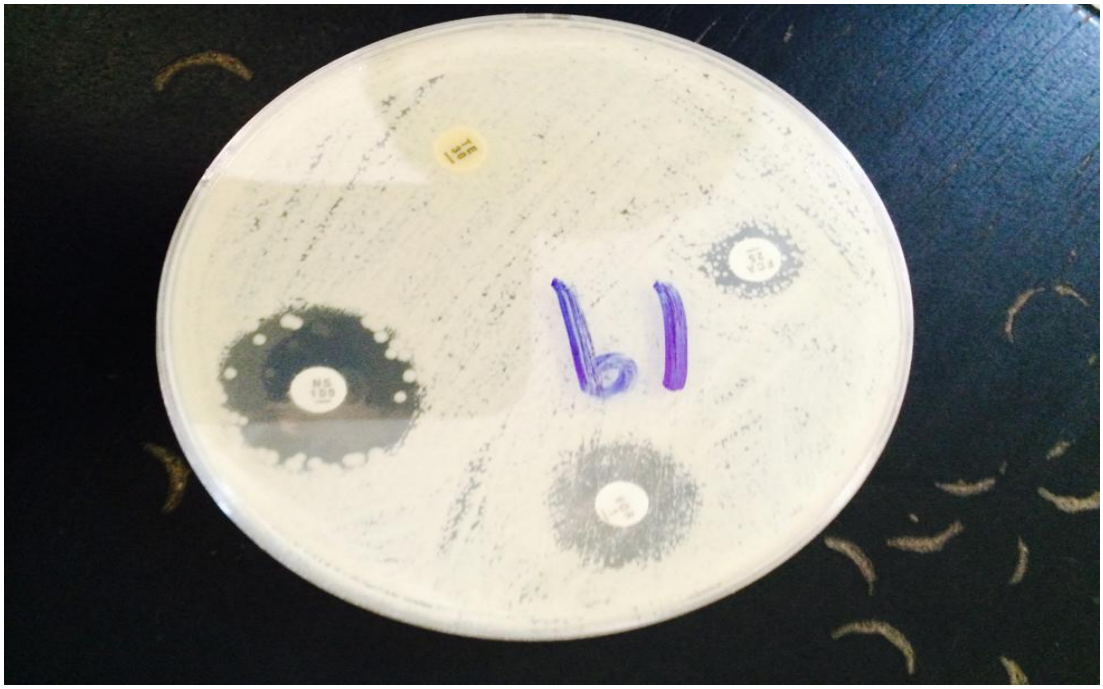
x.*Candida albicans*: Chlamydospore production on cornmeal-tween 80



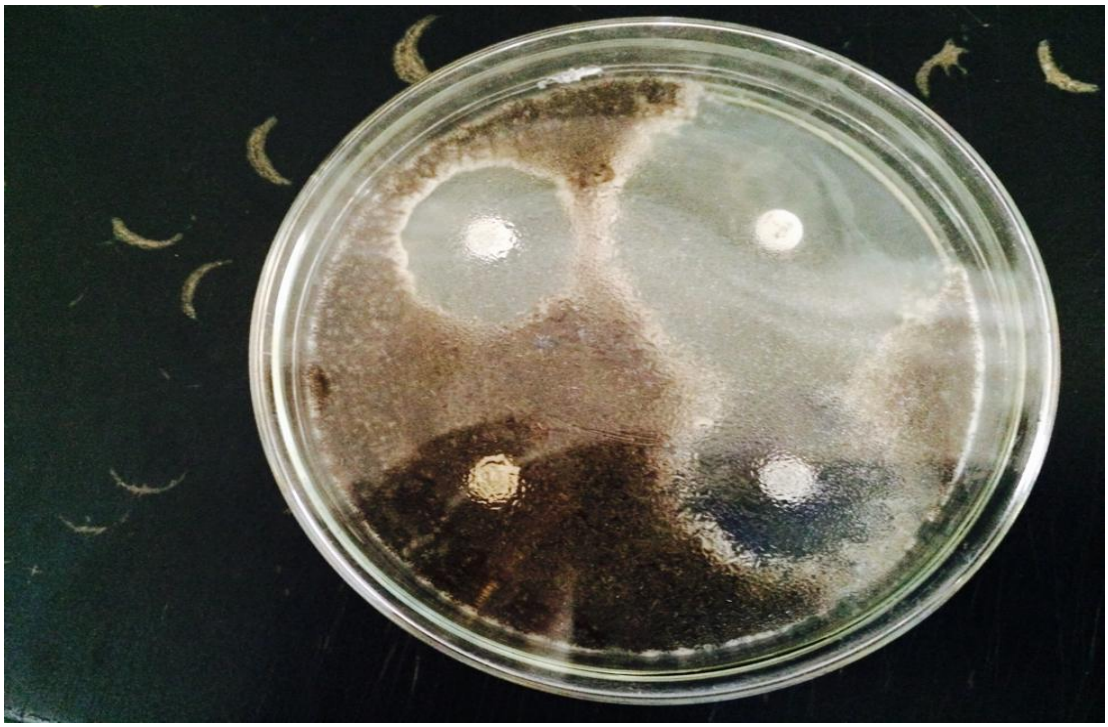
xi. Germ tube formation of *Candida albicans* in human serum



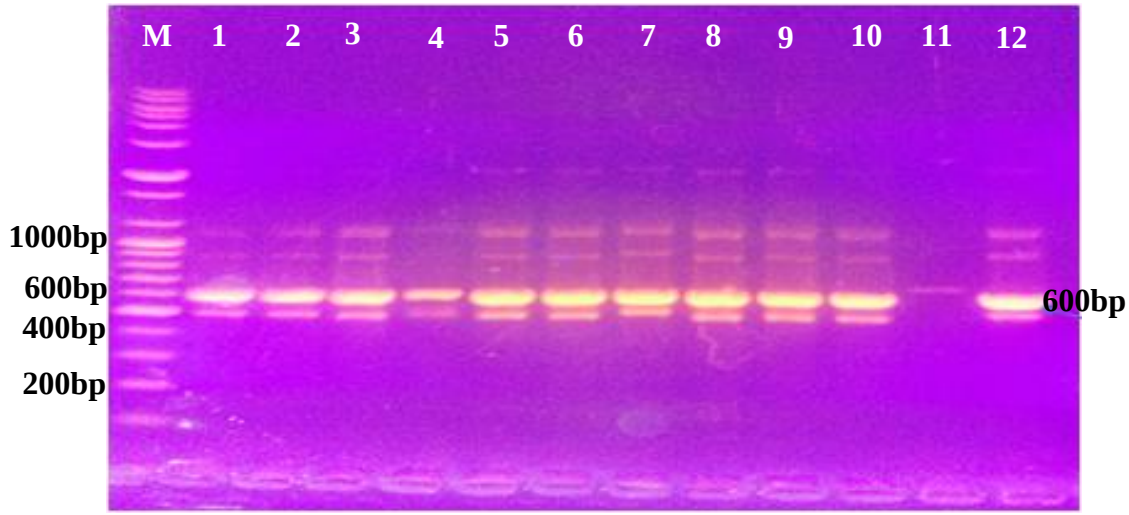
xii. API 20C AUX Kit used for yeast biochemical test



xiii. Antimycotic sensitivity testing for yeast



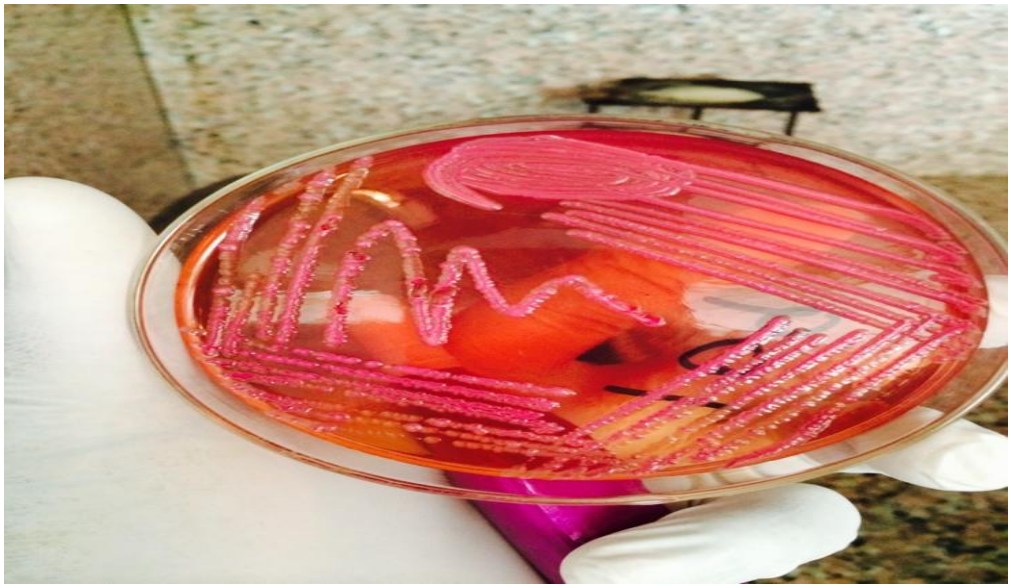
xiv. Antimycotic sensitivity testing for mold



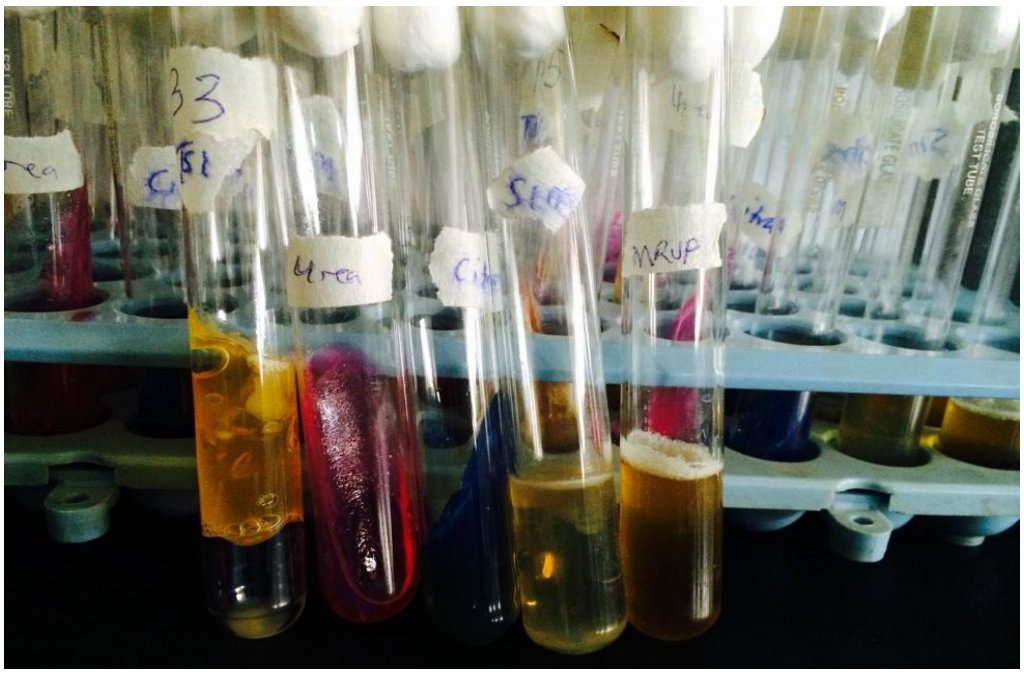
xv. Nested PCR of yeast isolates' DNA in bovine milk showing amplified D1/D2 domains of the 26S rRNA gene.

Seq->	S35	S34	S142	S155	S282	S38	S101	S201	S247	S54	S18	KM	KJ	KP	GS	JX	LC	KP0
S35	ID	0.584	0.736	0.439	0.739	0.733	0.682	0.733	0.739	0.682	1.000	0.324	0.325	0.324	0.332	0.337	0.332	0.333
S34	0.584	ID	0.416	0.299	0.417	0.411	0.417	0.411	0.417	0.417	0.584	0.198	0.199	0.198	0.203	0.205	0.201	0.201
S142	0.736	0.416	ID	0.531	0.996	0.987	0.930	0.987	0.996	0.930	0.736	0.347	0.347	0.347	0.308	0.365	0.364	0.362
S155	0.439	0.299	0.531	ID	0.531	0.527	0.525	0.527	0.531	0.525	0.439	0.275	0.275	0.275	0.298	0.258	0.258	0.258
S282	0.739	0.417	0.996	0.531	ID	0.990	0.930	0.990	1.000	0.930	0.739	0.347	0.348	0.347	0.308	0.366	0.364	0.362
S38	0.733	0.411	0.987	0.527	0.990	ID	0.921	1.000	0.990	0.921	0.733	0.347	0.348	0.347	0.300	0.366	0.364	0.362
S101	0.682	0.417	0.930	0.525	0.930	0.921	ID	0.921	0.930	1.000	0.682	0.335	0.335	0.335	0.298	0.352	0.351	0.349
S201	0.733	0.411	0.987	0.527	0.990	1.000	0.921	ID	0.990	0.921	0.733	0.347	0.348	0.347	0.300	0.366	0.364	0.362
S247	0.739	0.417	0.996	0.531	1.000	0.990	0.930	0.990	ID	0.930	0.739	0.347	0.348	0.347	0.308	0.366	0.364	0.362
S54	0.682	0.417	0.930	0.525	0.930	0.921	1.000	0.921	0.930	ID	0.682	0.335	0.335	0.335	0.298	0.352	0.351	0.349
S18	1.000	0.584	0.736	0.439	0.739	0.733	0.682	0.733	0.739	0.682	ID	0.324	0.325	0.324	0.332	0.337	0.332	0.333
KM	0.324	0.198	0.347	0.275	0.347	0.347	0.335	0.347	0.347	0.335	0.324	ID	0.997	1.000	0.297	0.795	0.791	0.795
KJ	0.325	0.199	0.347	0.275	0.348	0.348	0.335	0.348	0.348	0.335	0.325	0.997	ID	0.997	0.297	0.798	0.788	0.792
KP	0.324	0.198	0.347	0.275	0.347	0.347	0.335	0.347	0.347	0.335	0.324	1.000	0.997	ID	0.297	0.795	0.791	0.795
GS	0.332	0.203	0.308	0.298	0.308	0.300	0.298	0.300	0.308	0.298	0.332	0.297	0.297	0.297	ID	0.308	0.308	0.308
JX	0.337	0.205	0.365	0.258	0.366	0.366	0.352	0.366	0.366	0.352	0.337	0.795	0.798	0.795	0.308	ID	0.987	0.993
LC	0.332	0.201	0.364	0.258	0.364	0.364	0.351	0.364	0.364	0.351	0.332	0.791	0.788	0.791	0.308	0.987	ID	0.993
KP0	0.333	0.201	0.362	0.258	0.362	0.362	0.349	0.362	0.362	0.349	0.333	0.795	0.792	0.795	0.308	0.993	0.993	ID

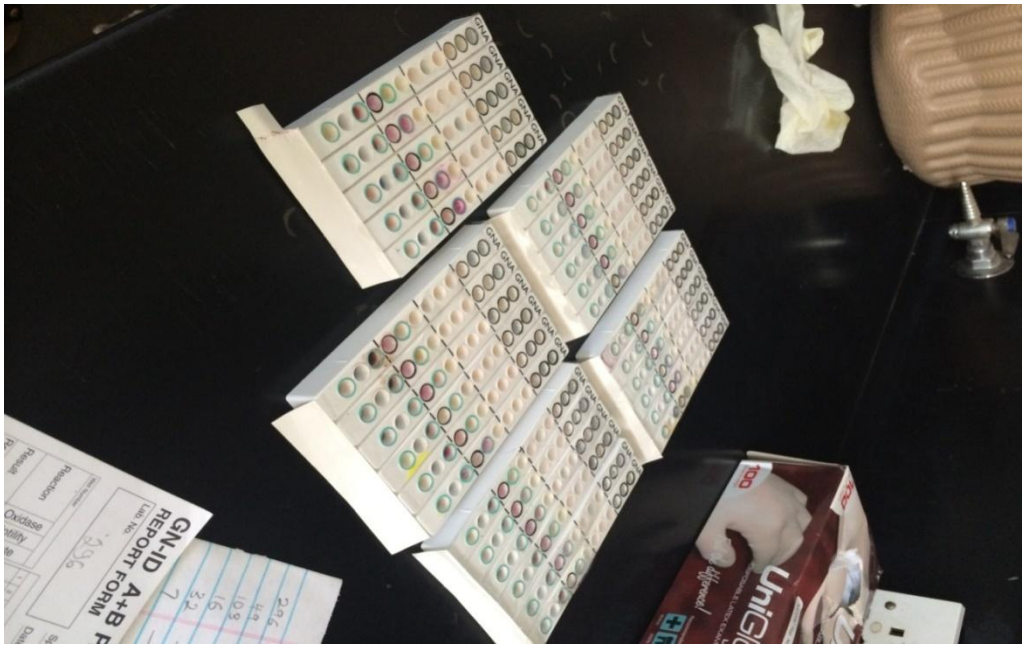
xvi. Nucleotide sequence identity matrix of fungi isolates from cow milk in Kaduna State.



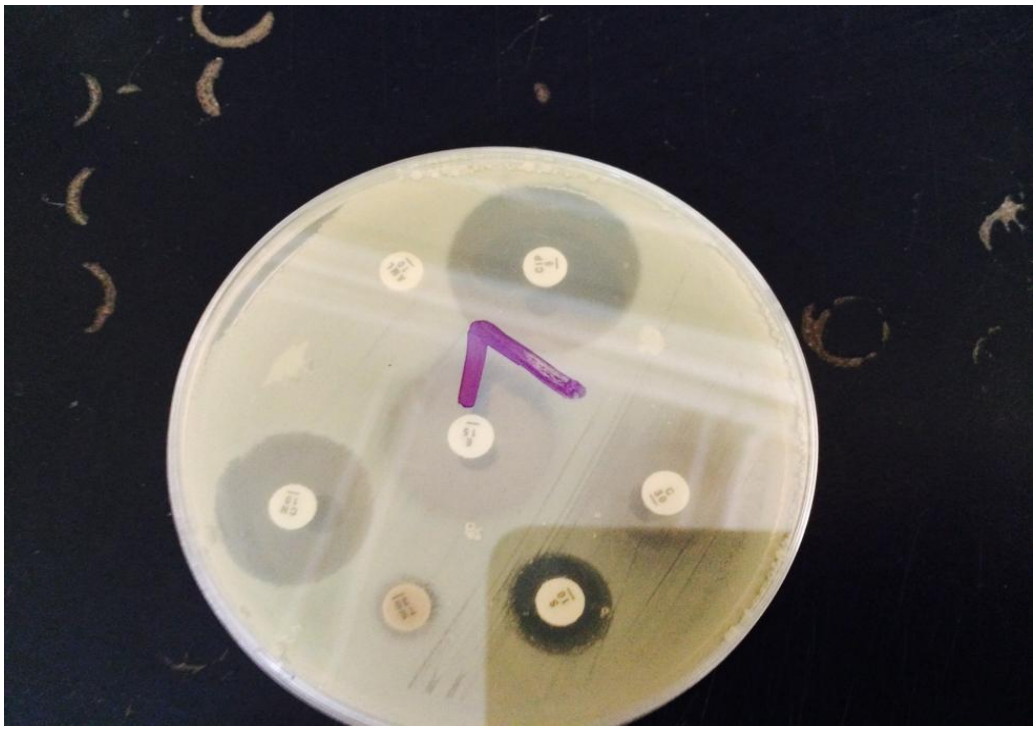
xvii. Colonies of coliform grown on MacConkey



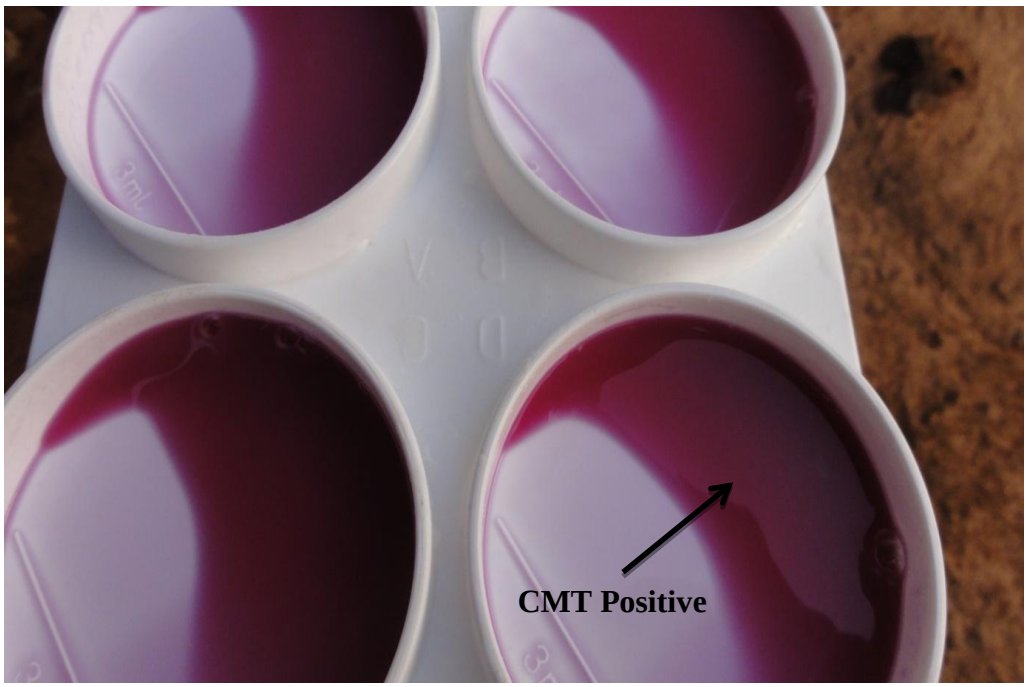
xviii. Conventional biochemical tests for *Enterobacteriaceae*



xix. Microgen Gram Negative ID Panels for identification of *Enterobacteriaceae*


xx. Antibiotic sensitivity testing for coliforms isolated from cow milk

xxi. CMT ofmilk sample from a cow with one blind quarter



xxii. Paddle showing CMT positive