

Full Length Research Paper

Molecular identification of yeasts associated with raw cow milk from peri-urban farms in Kaduna State, Nigeria

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This study aimed to examine the diversity of yeasts associated with bovine mastitis by employing molecular techniques in yeast identification. A total of 300 composite milk samples were collected from 26 peri-urban farms and examined. After culture, 37 (12.3%) fungal isolates were identified using the API 20C AUX. Polymerase chain reaction (PCR) and sequencing of the domains D1 and D2 of the 26S rRNA gene identified eleven (11) of these yeasts as *Candida albicans* (3 isolates), *Saccharomyces cerevisiae* (1 isolate) and *Pichia kudriavzevii* (7 isolates). The D1/D2 26S rRNA gene sequences were 100% identical for the yeast isolates within the same species. It was found that all *C. albicans* had ability to produce germ tubes by incubation on human serum and chlamydospores. Antimycotic sensitivity showed that 78% fungal isolates were sensitive to amphotericin B. The presence of isolates of the pathogenic yeast, *C. albicans* in this study raises the possibility of cow milk being a vehicle of transmission of pathogenic yeasts.

Key words: Bovine, milk, mastitis, yeasts, molecular identification.

INTRODUCTION

Bovine mycotic mastitis is usually caused by yeasts, but mastitis due to filamentous fungi, mostly *Aspergillus fumigatus* has been reported. 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).

Studies have shown that mycotic mastitis was on the increase, and the most frequently isolated organisms are *Candida* species (Tarfarosh and Purohit, 2008; Spanamberg et al., 2008) which are a group of unicellular opportunistic organisms, ever present in the natural surroundings of dairy cattle (milker's hands, milking

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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 (Seker, 2010). Some intramammary fungal organisms 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 (Seker, 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 lead to immune suppression and development of multiple drug resistant strains to compounds used in human medicines (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 mycotic species 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 PCR and DNA sequencing. This study draws attention to the importance of the disease in Nigeria, and could therefore stimulate interest for further investigations.

MATERIALS AND METHODS

Study design

A cross-sectional study was carried out on 26 farms in Kaduna State, Nigeria targeting peri-urban farms that provide milk to the community. A total of 300 milk samples were collected from apparently healthy cows and cows with clinical mastitis from four Local Government Areas. Selection of farms was based on: different management conditions, willingness of the farms/pastoralists to participate in the study, and accessibility of the location, so that samples collected could be immediately transferred 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 treatment before sampling.

Collection of samples

The teats of cows to be sampled were disinfected using a disposable paper towel immersed in 70% ethyl alcohol. The first stream of milk was allowed to flow out before volumes of about 10 ml (composite) were aseptically collected into labeled sterile universal bottles. The samples were kept in a cool box containing ice packs and transported to the laboratory immediately.

Enumeration and isolation of yeasts

A tenth of a milliliter of the milk sample to be tested (10^6) was directly inoculated on Sabouraud dextrose agar and evenly spread out with an 'L' shaped sterile glass rod. Plated culture media were incubated at room temperature for up to 72 h; representative colonies were stained on glass slides with lactophenol cotton blue. Then, all fungal isolates were purified by subculturing onto SDA plates containing gentamicin and incubated at room temperature for 72 h. Thereafter, individual colonies were picked and stored on SDA slants in McCartney bottles until ready to use.

Morphological studies of *Candida albicans*

Chlamydospore production

A plate of corn meal-tween 80 (Chlamydospore agar) was inoculated by lightly 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 chlamydo-spores borne on the tips of pseudohyphae.

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 h. 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 or absence of germ tubes.

Biochemical tests

The API 20C-AUX, a commercial yeast characterization kit (BioMerieux Vitek, France, product code 07221 C) was used according to the manufacturer's instructions for yeast identification on the basis of metabolic/physiological features. Identification was obtained by referring to the identification software.

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 and the inoculum was standardized to 0.5 McFarland and incubated for 4 h. 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 to streak the entire surface of the SDA before placing antimycotic discs 15 mm apart. The plates were incubated at 35°C and read after 24 h but for some strains where insufficient growth occurred, the plates were read after 48 h.

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

PCR reactions

Yeast cells grown on SDA were collected with the tip of a tooth pick from 48 h old colonies and DNA extracted using phenol-chloroform method. PCR reactions consisted of an initial step at 94°C for 7 min, 30 cycles of 94°C for 45 s, 59°C for 1 min, and 72°C for 30 s, with a final extension of 7 min at 72°C. Amplification of the 5.8S-ITS rDNA region was achieved with the primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Amplification of the D1/D2 domains of the 26S rRNA gene was done with the primers: NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') (White et al., 1990; El-Sharoud et al., 2009).

Gel electrophoresis

Gel electrophoresis was run for about an hour. Band sizes were estimated by comparison against 100 bp DNA ladder. The amplicons were viewed, 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.

DNA sequencing and phylogenetic analysis

For the direct confirmation of specificity of the PCR products, the DNA fragments generated by each 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 h. 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 and then subjected to GenBank BLASTN (Yeast-id database of the National Center for Biotechnology Information (NCBI) (blast.ncbi.nlm.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 *C. 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.

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 and sensitivity to antimycotics were compiled in tabular forms. Prevalence was calculated as number of yeasts isolated as the numerator with total number of isolates as denominator.

RESULTS AND DISCUSSION

In the present study, 37 yeast isolates were cultured from 300 milk samples and identified as: *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%) using API 20 C Aux (Table 1). Some literatures have reported the most common aetiological factors of mycotic mastitis to be the following genera of yeasts: *Candida*, *Cryptococcus*, *Rhodotorula*, *Trichosporon*, *Torulopsis* and *Geotrichum* (Krukowski et al., 2000; Krukowski et al., 2006 and Spanamberg et al., 2008). In tropical countries, more species of yeast-like fungi, as well as mildew fungi (*Aspergillus*, *Penicillium*, *Epicoccum*, *Phoma* and *Alternaria*) are found in the infected mammary secretions (Zur et al., 2002; Władysław et al., 2010).

The percentage isolation frequency of fungi in this study was 12.3%, with 6.7% of isolates belonging to the genus *Candida*. The rate of *Candida* spp. isolation was higher than other yeast and yeast-like organisms (5.6%) (Table 1). This is in agreement with other studies, where *Candida* yeasts were the most commonly isolated aetiological agents of mycotic mastitis in cows with the most frequent and abundant species as: *C. albicans*, *Candida krusei* and *Candida kefyr* (Santos and Marin, 2005; Władysław et al., 2010). The percentage of fungal isolation in surveys carried out in many countries varies

Table 1. Identification of fungal isolates from 300 milk samples using API 20 C Aux kit.

S/N	Fungi	No. of isolate	Frequency (%)
1	<i>Candida albicans</i>	9	24.3
2	<i>Candida famata</i>	4	10.8
3	<i>Candida krusei</i>	4	10.8
4	<i>Candida boidinii</i>	1	2.7
5	<i>Candida pelliculosa</i>	1	2.7
6	<i>Candida lusitanae</i>	1	2.7
7	<i>Trichosporon mucoides</i>	5	13.5
8	<i>Cryptococcus humicola</i>	1	2.7
9	<i>Cryptococcus laurentii</i>	1	2.7
10	<i>Cryptococcus albidus</i>	1	2.7
11	<i>Saccharomyces cerevisiae</i>	2	5.4
12	<i>Stephanoascus ciferrii</i>	2	5.4
13	<i>Rhodotorula mucilaginosa</i>	2	5.4
14	<i>Kloeckera</i> spp.	1	2.7
15	<i>Kodamaea ohmeri</i>	1	2.7
16	<i>Geotrichum capitatum</i>	1	2.7
	Total	37	100

considerably, with as 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 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). *C. 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 chlamydo spores and germ tubes (hyphal formation) which are considered as confirmatory for identification of *C. albicans* (Yang, 2003; Zaini et al., 2006). The source of these pathogenic yeasts in milk is not well known; however, but 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 transmission vehicles of pathogenic yeasts (Fleet, 2007) but the present study 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 (Table 1), while PCR and sequencing identified 7 isolates

as *P. kudriavzevii* (Figure 1). This yeast is abundant in the environment and can be found in soil, fruits and various fermented beverages. So far, *P. kudriavzevii* is mainly associated with food spoilage, causing surface biofilms in low pH products. 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. However, *C. neoformans*, which is considered the most dangerous species, was not identified. But *C. laurentii* 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. *Cryptococcus* spp. has been reported as causing a rather severe clinical mastitis but since this study was concerned primarily with subclinical infection, fewer *Cryptococcus* isolates were expected. The cultural methods used may also have been more effective in isolation of *Candida* species.

Kodamaea ohmeri (*Pichia ohmeri*) was initially isolated in Brazilian artisanal cheese, cucumber salts (brines) used in the food industry for the fermentation of pickled foods, tree barks, fruits and marine fish (Borrelli et al., 2006; Li et al., 2008). It has also been isolated from environmental sources such as, pools, sand, floors and sea water (Chakrabarti, 2014). In the past, *Kodamaea ohmeri* was considered a contaminant, but at present some species are recognized as emerging opportunistic

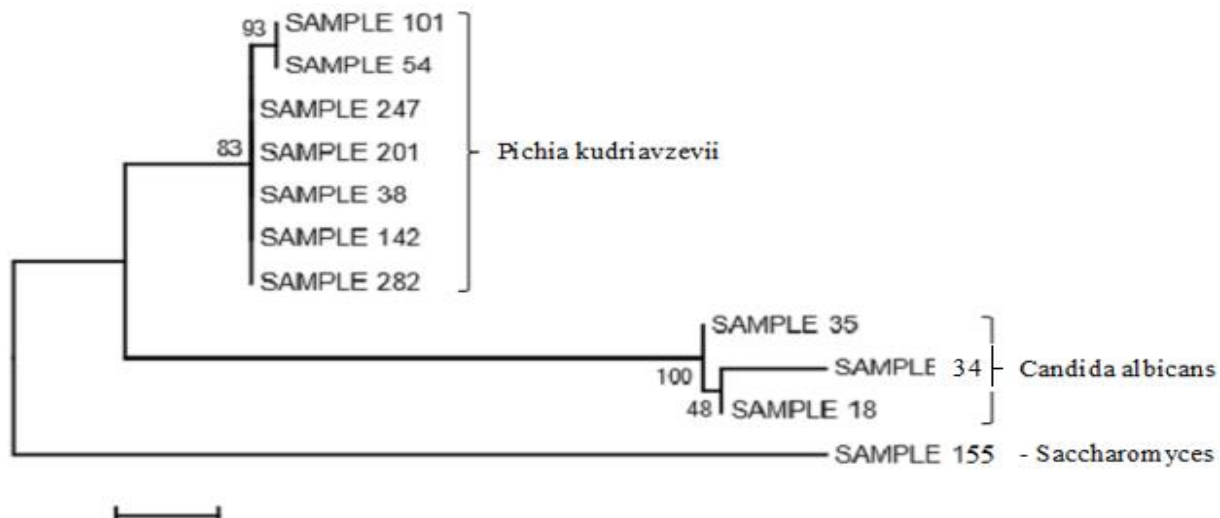


Figure 1. Midpoint neighbor-joining phylogeny tree analysis of the strains of fungi isolates from milk samples in Kaduna State.

pathogens 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 might have been influenced by the type of management system employed in the dairy farms studied. Although, it is not expected that these microorganisms can survive thermal treatment, milk may be a carrier for a great diversity of agents that could be harmful to public health (Krukowski et al., 2006; Kivaria and Noordhuizen, 2007).

Recent data indicate that, molecular techniques based on PCR have been used as a tool for diagnosis of several fungal species (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 isolates tested indicated the presence of 1 distinctive band (600bp) (Plate 1). The % identity from blast show that all the isolates identified as *Pichia kudriavzevii* (also known as *Candida krusei*) were between 97-99% identical to *Pichia* strain; the 3 *Candida* isolates show 95-98% identity with *C. albicans*, while only 1 *Saccharomyces cerevisiae* isolate was 91% identical with *Saccharomyces* strain from the blast (Table 2).

Phylogenetic reconstruction based on the D1/D2 26S rRNA gene sequences of the 11 isolates grouped them in 3 clusters (Figure 1). Clusters 1 and 2 showed that all the isolates (7) identified as *P. kudriavzevii* formed a monophyletic group with 93-100% nucleotide identity

among themselves and were most closely related to two of the isolates identified as *C. albicans* with a 73.3-73.9% nucleotide identity. However, their nucleotide identity with one of the *C. albicans* strain was between 41.1-41.7%. Cluster 3 showed that out of the three *C. albicans* strains identified, two were 100% similar but were not as identical as the other *C. albicans* isolate in the same monophyletic 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%). Comparison of the isolates in this study with those from the GenBank showed that the sequences of the newly identified isolates seems to be different from the available sequences accessed with 19-36% nucleotide identity (Figure 2).

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 (Figure 2). 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. lusitanae* 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 (78.1%) of the fungi isolates were sensitive to amphotericin B while, 24 (77.4%) were sensitive to griseofulvin and 13 (42%) to nystatin (Table 3). In terms of decreasing efficacy of antimycotics on isolates used in this study, antimycotics can be ranked as follows: Amphotericin B > Griseofulvin

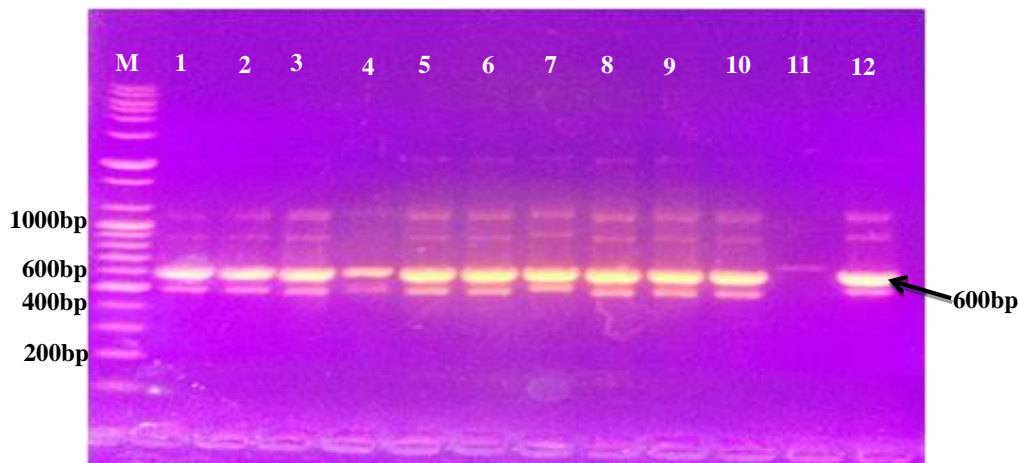


Plate 1. PCR of yeast isolates' DNA in bovine milk showing amplified D1/D2 domains of the 26S rRNA gene. M= 100 bp ladder, Lane 1 and 2 = *Candida albicans*, Lane 4 to 6 = *Pichia kudriavzevii*, Lane 6 to 10 = *Saccharomyces cerevisiae*, Lane 11 = negative control, Lane 12 = positive control.

Table 2. GenBank accession numbers of sequences of 26S rRNA genes of fungal isolates used for phylogenetic analysis.

Fungi designation	ID (%)	Country of origin	Sampling year	GenBank accession no.
<i>Candida albicans</i>	95	China	2014	KM013360
<i>Candida albicans</i>	98	South Africa	2016	KJ534505
<i>Candida albicans</i>	98	USA	2015	KP780462
<i>Pichia kudriavzevii</i>	97	Iran	2009	GS599266
<i>Pichia kudriavzevii</i>	98	Italy	2012	JX129895
<i>Pichia kudriavzevii</i>	99	Brazil	2014	LC015306
<i>Saccharomyces cerevisiae</i>	91	South Africa	2014	KP070744

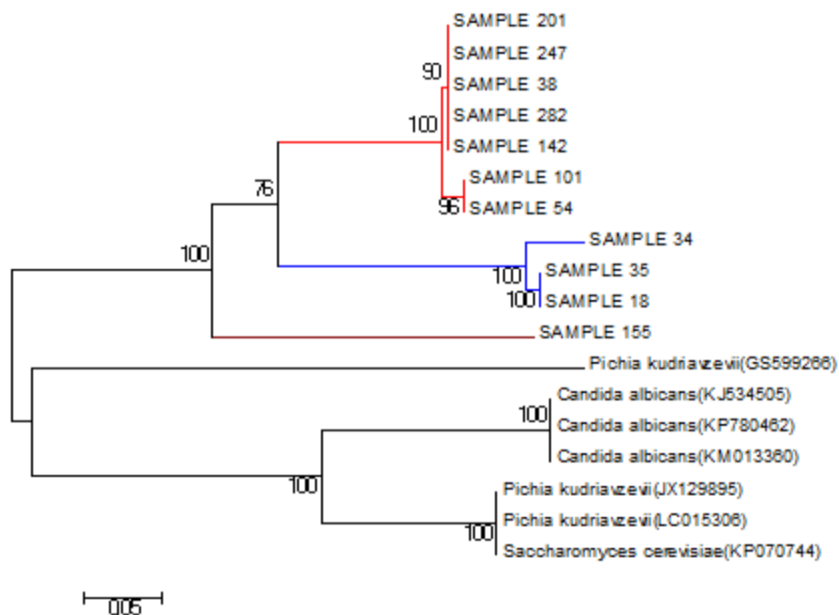


Figure 2. Midpoint neighbor-joining phylogeny tree analysis of the strains of fungi isolates from milk samples in Kaduna State compared with available sequences of fungi strains from the GenBank.

Table 3. *In vitro* sensitivity testing of fungal isolates.

Fungal species	No. of isolates	No. (%) sensitive to each antimycotic				
		NYS	VOR	FLU	GRI	AMP
<i>Saccharomyces</i> spp.	2	1(50)	1(50)	1(50)	1(50)	1(50)
<i>Candida</i> spp.	20	7(35)	0	0	17(85)	17(85)
<i>Kodamea</i> spp.	1	0	0	0	0	0
<i>Stephanoascus</i> spp.	2	1(50)	1(50)	0	2(100)	2(100)
<i>Cryptococcus</i> spp.	3	0	0	0	1(33.3)	3(100)
<i>Rhodotorula</i> spp.	2	2(100)	2(100)	1(50)	0	1(50)
<i>Trichosporon</i> spp.	5	1(20)	1(20)	0	2(40)	4(80)
<i>Geotrichum</i> spp.	1	1(100)	1(100)	0	0	0
<i>Kloeckera</i> spp.	1	0	0	0	1(100)	1(100)

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

> Nystatin > Variconazole > Fluconazole.

Conclusions

This study has showed that diverse yeasts existed in the examined milk samples from peri-urban dairy farms and confirms the involvement of yeast in some of the cases of mastitis in dairy cows. Also, this study reports the 1st incidence of the pathogenic yeast, *C. albicans* in cow milk in Nigeria and that milk could act as a transmission vehicle of this yeast organism.

Conflict of Interests

The authors have not declared any conflict of interests.

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