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Molecular Identification, Virulence Factors and Antifungal Resistance among *Pichia kudriavzevii* Isolated from Urine of Patients on Catheter

Oluwole Moses David[1*], Omowunmi Omobolaji Adelowo[1], Omoyeni Theophilus[1], Oluwaseun Nancy Adetan[1], Tosin Ojo-Bola[2], John Babatope Owabumoye[1] and Dauphin Dighitoghi Moro[3]

[1] *Department of Microbiology, Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria*
[2] *Department of Medical Microbiology and Parasitology, Federal Teaching Hospital, Ido-Ekiti, Nigeria*
[3] *Department of Microbiology, Lagos State University, Ojo, Lagos State, Nigeria*

Abstract

Pichia kudriavzevii is commonly used in food processing, its association with human infection is very rare. In the few cases where it has been reported, it is rarely present in healthy individuals. This study investigated the incidence of *Pichia kudriavzevii* among patients on catheter in a tertiary institution in Ekiti State. A structured questionnaire was used to collect demographic data of the patients. Fifty-six non-repeat catheterized urine samples were collected from consented patients and both pathogenic factors and antifungal resistance were determined among the *Pichia kudriavzevii* isolated. Out of the 56 urine samples tested, 15 (26.8%) were positive for *P. kudriavzevii* while 31 (73.2%) showed growth of other fungi, mostly of the genus *Candida*. Out of the antifungal agents tested against *P. kudriavzevii*, ketoconazole [n=11 (73.3%)] was the most effective followed by clotrimazole [n=7 (46.7%)] and nystatin [n=6 (40%)]. According to the outcomes of the present study, *P. kudriavzevii* appears to be associated with urinary catheter and with many virulence factors. The isolates were also resistant to first-line antifungal drugs in addition to the virulence factors. This yeast may emerge as another fungal pathogen especially among immunocompromised.

Keywords: *Pichia kudriavzevii,* catheter, urine, yeast, antifungal

Резюме

Дрождите от вида *Pichia kudriavzevii* обикновено се използват при преработка на храни, но е известна и връзка им с инфекция при хора. В малкото докладвани случаи, този вид рядко се среща при здрави индивиди. Настоящото проучване изследва честотата на *P. kudriavzevii* сред пациенти с катетър настанени в третостепенна болница в щат Ekiti, като за събиране на демографските данни е използван структуриран въпросник. От 56 съгласни катетеризирани пациенти са събрани проби от урина, след което са определени патогенните фактори и антифунгалната резистентност на изолатите от *P. kudriavzevii*. От всички 56 тествани проби, 15 (26.8%) са положителни за *P. kudriavzevii*, докато останалите 31 (73.2%) показват развитие на други гъби, предимно от род *Candida*. Най-голям ефект от антифунгалните препарати показва кетоконазол [n=11 (73.3%)], последван от клотримазол [n=7 (46.7%)] и нистатин [n=6 (40%)]. Резултатите от настоящото проучване доказват връзката на че P. Kudriavzevii с уринарните катетри и факторите на вирулентност. Изолатите също са резистентни към антифунгални лекарствени средстав от първа линия в допълнение към факторите на вирулентност. Тези дрожди могат да се появят като допълнителен гъбичен патоген, особено сред имунокомпрометирани пациенти.

* Corresponding author: david.oluwole@eksu.edu.ng

Introduction

Urethral catheterization is used for patients of all ages but it is very common among the elderly and chronically ill patients (Hollingsworth *et al.,* 2013). Indwelling urinary catheters have been reported as the leading cause of nosocomial infections. Catheterization is responsible for the high incidence of urinary tract infections (UTI). *Candida* species are the most commonly encountered opportunistic fungi that can colonize urethral catheters (Kauffman, 2005; Padawer *et al.,* 2015).

Pichia kudriavzevii, a teleomorph of *Candida krusei*, is a yeast extensively distributed in the environment. *P. kudriavzevii* and *C. krusei* have been reported to be the two names used for the same organism. Approximately 99.6% of their DNAs are similar while they still share other attributes. *P. kudriavzevii* has been reported to have a wide application in beverage (e.g nunu, wine) and food (e.g. cassava, sourdough bread) fermentation processes (Akabanda *et al.,* 2013**;** Yuangsaard *et al.,* 2013; Del Mónaco *et al.,* 2014). However, its recent implication in different human diseases is worth noting. The telemorph of the organism, *C. krusei,* has ranked as the one of the most common cause of candidemia and duodenal perforation (Sridhar *et al.,* 2006; Al-Bshabshe *et al.,* 2019**)**. It is also a major cause of invasive fungal infections among patients undergoing grafting and those with haematological disorder.

P. kudriavzevii has been repeatedly isolated from infant diarrhea and regularly from other human diseases. The species is very difficult to isolate because of its strong similarities to already established and popular species of the *Candida* genus (Ellis, 2016). This makes its correct identification critical and of serious clinical significance, and presents a very good basis for proper diagnoses and treatment. In most cases, administration of antifungals commences immediately after sepsis yields positive fungal growth (Lockhart *et al.,* 2017). The presence of the *P. kudriavzevii* in catheterized urine may be due to contamination of the device itself or introduced during the process. The aim of this study is to determine the association of *P. kudriavzevii* with the urine of patients on catheter. The antibiotics susceptibility of the isolate and the incidence of the virulence factors in the isolates were also determined.

Materials and Methods

Collection of samples

Fifty-six non-repeat catheterized urine samples were collected aseptically in a tertiary hospital in Ekiti State, Nigeria. The samples were collected from 36 males and 20 females of different ages as described by Shepherd (2017). The catheter tube was stabilized by holding it below the level of the sampling port and the tip of a sterile syringe was inserted into the sampling port earlier disinfected with 70% alcohol. The mouth of a sterile universal bottle was wiped with 70% alcohol-impregnated swab and allowed to dry. Approximately 10 mL of urine sample was withdrawn into it and the bottle was labelled immediately. The samples were taken to the laboratory within one hour of collection. The samples were plated on sterilized Sabouraud Dextrose Agar (SDA) (LabM) supplemented with chloramphenicol and incubated at 37°C for 48 h. Colonies with shiny appearance were sub-cultured on the same medium and incubated at the same conditions. Colonies that were cream-colored, raised, entire, smooth and butyrous on SDA were picked and inoculated into SDA slants.

Characterization of isolates

The ability of the isolates to utilize and assimilate different sugars which included galactose, glucose, lactose, maltose, rafinose, sucrose and trehalose was tested. The isolates were also grown in 5% glucose broth containing 10% NaCl and incubated at 37°C for 24 h. The ability of the isolates to grow at elevated temperature (40°C) was also determined. Dalmau plate method was used to determine the ability of the isolates to produce pseudohyphae. A corn meal agar plate was streaked with copious pure isolates using a wire loop and a sterile glass cover slip was placed over a part of the streak. The plate was examined directly on a microscope each day for about three days through the cover glass and tendency to produce hyphae, pseudo-hyphae or conidia was noted. Urase utilization by the isolates was determined as described by Fawole and Oso (2004). Two distinct colonies were introduced into a medium containing 0.1% dextrose, 0.1% peptone, 2% urea, 0.5% NaCI, 0.2% KH_2PO_4 and phenol red as indicator. Development of a red-pink color indicated a positive result as reported by Fawole and Oso (2004).

DNA extraction

The DNA of the isolates tentatively identified using cultural means were extracted using the Qiagen DNA tissue kit (Germany). The extracted DNA was stored at -20°C for further use. Amplification of the ITS1-5.8S-ITS2 region was done by universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG

G -3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) at the annealing temperature of 56°C. The amplification was done for 35 cycles of 98°C for 30 s and annealing temperatures of 60°C and 72°C both for 30 s. This was followed by a final extension of 72°C for 5 min. The nucleic acid sequencing was done by Macrogen. The nucleic acid sequences were compared with the database at the GenBank database using the BLAST sequence search tool. The phylogenetic relationships of the isolates were constructed by plotting a phylogenetic tree with the nearest neighbour joining method using MEGA 6.0 software.

Determination of virulence factors

Phospholipase test. Approximately 10% sterile egg yolk was introduced into sterilized SDA and the isolates were radially streaked on it. The culture was incubated at 37°C for 48 h. Whitish coloration seen around the organism was recorded as positive for phospholipase production.

Proteinase test. The method of Mohan and Ballal (2008) was used to detect the ability of the isolates to produce proteinase. Approximately 0.2 % bovine serum albumin was incorporated into SDA. The isolates were radially streaked on the agar and incubated at 37°C for 3 to 6 days. Hazy area surrounding the growth on the agar was recorded as positive.

Haemolysis. The protocol by Luo *et al*. (2002) was used to determine haemolysin production among the isolates. Sabouraud dextrose agar was supplemented with 6% human blood and 3% glucose (pH=5.6) and a loopful of standardized inoculum was inoculated on the plate at 37°C for 5 days and the result was read.

*Gelatinase production***.** Sterilized Sabouraud dextrose agar supplemented with 1% gelatin as described by Ramesh *et al*. (2011). The plate was then incubated for 5 days at 37°C. The appearance of an inhibition zone was clearly visualized by the addition of 0.1% mercuric chloride. A hazy zone around the colony was recorded as a positive result.

Biofilm production. Congo Red Agar (CRA) was prepared by supplementing SDA with sucrose (5%) and Congo red (0.8 g/L), according to Mathur *et al.* (2006). Isolates were streaked on agar and incubated aerobically for 24 to 48 h at 37°C. Positive results manifested as black colonies with dry crystalline consistency (Pallavi *et al.,* 2016).

Antifungal sensitivity pattern. Three antifungals were tested against the isolates with the followings concentrations in µg: clotrimazole (10) ketoconazole (10) and nystatin (100 units) as described by CLSI (2012). The zone of inhibition was measured and interpreted accordingly.

Results

Laboratory data of 56 patients, whose specimens were obtained and evaluated, were all on urinary catheter. Table 1 shows the demographic distributions of patients by sex, age, and number of samples that yielded fungal growth on SDA. The results also revealed that 15 (26.76%) patients were between the age of 28 and 40 years, 26 (46.43%) between 41 and 60 years, and 22 (39.29%) were older than 60 years. It was observed that 63.67% (n=36) of the patients were male and 33.33% (n=20) were female. A total of 45 (80.36%) out of 56 urine samples yielded fungal growth on SDA while 11 (19.64%) did not show fungal growth.

Table 1. Demographic studies of specimens collected from a tertiary health facility in Ekiti State, Nigeria

Attributes	Number of Isolates	[%]
Ages distribution		
28-40	15	26.79
41 -60	26	46.43
60 and above	22	39.29
Total	56	100.00
Sex		
Male	36	63.67
Female	20	33.33
Total	56	100.00
Growth on SDA		
Positive for fungi growth	45	80.36
Negative for fungi growth	11	19.64
Total	56	100.00

P. kudriavzevii were recovered from 15 (33.33%) out of 45 samples that yielded fungal growth among the samples collected for this study. Thirty (66.67%) of the recovered fungi were mostly of the genus *Candida*. The biochemical and morphological characteristics of the isolates (*P. kudriavzevii*) produced tannish-white, dull, butyrous, low convex colonies with a flattened center, varying margin smooth to lobed, and fringed colonies. The colonial morphology appears to be butyrous and light cream in colour. Examined under microscope, the cells appeared to be ovoid to elongate in shape and occurred mainly singly and in pairs. The pseudohyphae observed were abundant and moderately

branched. All the isolates were able to utilize none of the sugars except glucose. In the medium containing glucose they showed a sign of fermentation.

The PCR amplification of ITS gene of the fungal isolates which were tentatively identified using cultural and morphological methods. Lane M and negative (-ve) are markers and negative lanes, respectively, while lanes 1-6 are DNA of the fungal isolates from this study. The DNA of the isolates was about 500 bp as shown in Fig. 1. The phylogenetic relatedness of the isolates based on nucleic acid sequence using Neighbor-Joining method is shown in Fig. 2.

Twelve of the isolates produced biofilm and phospholipase apiece, while nine of the isolates produced proteinase. Proteinase has the least occurrence among the virulence factors (Table 2). A total of 8, 4 and 9 of the isolates were resistant to clotrimazole, ketoconazole and nystatin, respectively, as shown in Table 3.

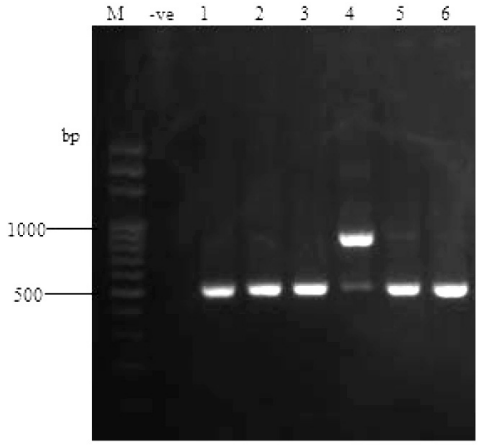

Fig. 1. The result of amplification of genomic DNA of the isolates using ITS1 and ITS4 primers. Lanes 1 and 2 represent marker and the negative control while other lanes are the isolates from this study.

Table 2. Incidence of virulence factors in *P. kudriavzevii* isolated from patients on catheter

Virulence Factor	Condition	
	Positive (%)	Negative (%)
Biofilm	12 (80.00)	3 (20.00)
Gelatinase	10 (66.67)	5 (33.33)
Haemolysis	10 (66.67)	5 (33.33)
Phospholipase	12 (80.00)	3 (20.00)
Proteinase	9 (60.00)	6 (40.00)

Table 3. Antifungal susceptibility *P. kudriavzevii* isolated from catheterized urine

Antifungals	Resistance	Susceptible
Clotrimazole	8 (53.33)	7 (46.67)
Ketoconazole	4 (26.67)	11 (73.33)
Nystatin	9 (60.00)	6 (40.00)

Discussion

Urinary tract candidiasis is known as the most frequent nosocomial fungal infection worldwide. In this study, the organism was observed to be present in 26.79% of the total samples collected. This represents a very high proportion and may be due to the prophylactic usage of fluconazole and other azole antifungals to which the isolates are basically resistant. *P. kudriavzevii* has very poor constitutive expression of genes that aids efflux pumps (Deresinski, 2018). Predisposing factors like advanced age, length of hospital stay, surgery and diabetes mellitus contribute to the high prevalence of fungal colonization and infection (Leuck *et al*., 2012). Rishpana and Kabbin (2015) also stated that the longer patients are on urinary catheterization, the more the chance of fungal colonization. This may

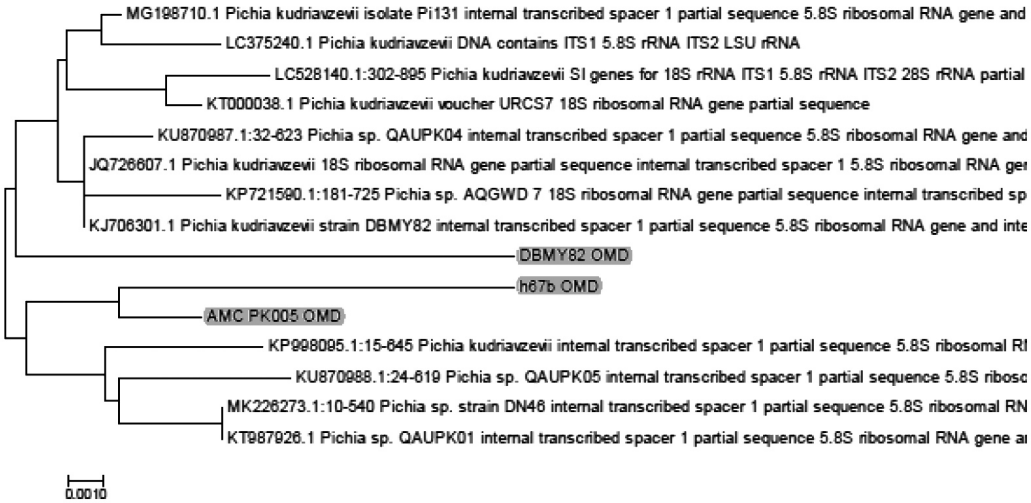

Fig. 2. A phylogenetic tree based on nucleic acid sequence of selected isolates in relation to closely related yeast species using the Neighbor-Joining method

also increase the population and heterogeneous nature of the fungal population. Reports have shown that both male and female urinary tracts could be colonized by fungi (Colodner *et al.,* 2008; Rishpana and Kabbin, 2015).

Biofilm production has been reported to be a common characteristic of fungi associated with UTIs. Apart from the fact that the biofilm significantly contributes to the antifungal resistance of the fungi, it also aids attachment to the surface of catheters (Trautner and Darouiche, 2004; Tumbarello *et al.,* 2007). *P. kudriavzevii* has been reported to produce a characteristically strong biofilm that has high tolerance to heat and oxidative stresses (Giobbe *et al.,* 2007). The biofilm formed had higher tolerance to heat and oxidative stresses (Chi *et al.,* 2015). Resistance of uncommon yeast to antifungals should be given attention because of their unusually high MICs previous and possible misidentification. *P. kudriavzevii,* for instance, has no established susceptibility breakpoints for some common antifungals (Leuck *et al*., 2012; Taj-Aldeen *et al.,* 2014).

Non-*albicans Candida* is increasingly becoming a group of organisms to watch out for in clinical practice because of their involvement and association with different human infections. *Pichia kudriavzevii* has not been earlier reported as a common nosocomial pathogen, neither has its association with urinary tract infections in the study area. *P. kudriavzevii* isolated in this study possessed different virulence factors and at the same time are resistant to different antifungals. Results in this study establish *P. kudriavzevii* to be associated with catheterization and also possess different virulence factors and are at the same time resistant to antifungals. The generally regarded as safe' status of the yeast and its candidacy as a probiotic should be reviewed.

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