Research Article

PREVALENCE AND ANTIFUNGAL SUSCEPTIBILITY PATTERN OF *CANDIDA DUBLINIENSIS* ISOLATED FROM URINE SAMPLES

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ABSTRACT

In this study, 2, 617 urine samples were collected from the clinically suspected patients and examined the prevalence and antifungal susceptibility pattern of *Candida dubliniensis*. In this study, overall prevalence of *C. dubliniensis* is 0.34%.The phenotypic identification methods for *C. dubliniensis* included germ tube test, production of rough colonies and chlamydospores, determination of assimilation and fermentation, Hi*Candida* identification kit, morphology on Hichrome agar and growth at 45°C. Totally, 112 isolates were recovered from the urine samples and those were directly subjected to germ tube test. Out of which, 61 (54.46%) isolates were positive in germ tube production. Among 61 germ tube positive isolates, 9 were identified as *C. dubliniensis* with the prevalence of 14.75%. Antifungal susceptibility test was performed with amphotericin B, clotrimazole, fluconazole, itraconazole, ketoconazole, miconazole and nystatin. All the 9 isolates showed (100%) sensitive to amphotericin B, followed by fluconazole and ketoconazole (88.88%). This study highlighted the usefulness of Hichrome agar and temperature test (growth at 45°C) in presumptive identification of *C. dubliniensis* and combats the existing view that this species occur in particular anatomic sites of specific population through this species recovered from urine of HIV/AIDS negative individuals.

INTRODUCTION

Candida spp. are the most frequent source of fungal infections and it is responsible for diverse clinical manifestation from life-threatening to non-life-threatening diseases (Eggimann *et al*, 2003). Though it is a commensal in healthy individuals, in immunocompromised situations it may cause systemic infection due to their flexibility to different host niches. It is not always easy to find out when *Candida* is an "innocent bystander" or when it is playing a pathogenic role (Delaloye and Calandra, 2014). Worldwide, increased the occurrence of invasive infections those caused by *Candida* species has reported. At present, more than 150 species of *Candida* were recognized. But only a few species are most frequently associated with human infection. Among *Candida* species,

Candida albicans was historically identified as major species accounting for two-thirds of *Candida* infections worldwide. It has an adaptable morphogenesis: existing in three forms (i.e., yeasts, pseudohyphae and hyphae) and has two lifestyles (planktonic and biofilm), permitting *C. albicans* to thrive under diverse environmental conditions (Wong *et al*, 2014). Recently, the incidence of non-albicans *Candida* spp., like *Candida tropicalis*, *Candida parapsilosis* and *Candida glabrata* is increasing worldwide with increased drug resistance (Pfaller and Diekema, 2007).

In 1995, a new *Candida* species was identified from the oral cavity of HIV-infected individuals in Dublin, Ireland (Sullivan *et al*, 1998). *C. dubliniensis* has recently stated as a potential opportunistic pathogen and it is most frequently recovered from the oral cavities of HIV-infected patients (Linares *et al*, 2007).

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But it has also been isolated from HIV- and non-HIV patients (Pinjon *et al*, 1998; Meis *et al*, 1999; Salesa *et al*, 2001; Marriott *et al*, 2001; Sebti *et al*, 2001), from vaginal, urinary, fecal specimens (Sullivan *et al*, 1995; Odds *et al*, 1998; Quindós *et al*, 2000) and blood culture (Meis *et al*, 1999). Phenotypic identification and differentiation of *Candida dubliniensis* are still challenging in routine diagnosis. Due to the ineffective sampling and detection methods or the sampling on the wrong anatomic sites, the prevalence of *C. dubliniensis* among normal healthy individuals has been misjudged probably (Sullivan *et al*, 2005). Despite the effort that has been expended to identify *C. dubliniensis* in clinical samples, a definitive assessment of the prevalence of this species is still lacking (Sullivan *et al*, 2005). Therefore, the present study was aimed to determine the prevalence and differentiation of *C. albicans* and *C. dubliniensis* isolated from a urine sample of clinically suspected patients.

MATERIALS AND METHODS

The study included the clinical strains of *C. albicans* and *C. dubliniensis* recovered from urine samples of various hospitalized clinically suspected patients from Microbiology department at Doctor's Diagnostic Center, Tiruchirapalli, India. Further works were carried out in the Department of Microbiology, Vivekanandha College of Arts and Sciences for Women (Autonomous), Namakkal, Tamil Nadu, India. All the isolates were stored with glycerol on deep freezer until tested.

Urine sample

A total of 2, 617 urine specimens were collected from clinically suspected patients at Doctor's Diagnostic Center laboratory, Tiruchirapalli, Tamil Nadu, India. The collected samples were directly subjected to gram's staining, in addition, specimens were routinely cultured on blood agar, MacConkey and Cystine Lactose Electrolyte Deficient (CLED) agar (Himedia, Mumbai, India), for the recovery of microbial growth (bacteria and *Candida* species). Then the culture plates were incubated at 37°C for 24 to 48 h. After incubation, growth was observed and suspected colonies were inoculated on Sabouraud dextrose agar (SDA) plates supplemented with chloramphenicol (Himedia, Mumbai, India) and incubated first at 37°C for 24 to 48h to observe potential growth. Suspected colonies of *Candida* were confirmed by mycological investigation.

Isolation and differentiation of C. dubliniensis
KOH mount

For direct microscopic examination, the clinical sample was placed on the glass slide then added the 10% KOH (Potassium Hydroxide) and a coverslip was placed over the sample. Slide was slightly warmed and gentle pressure was given over the coverslip to remove the trapped air and then observed under the microscope using the low power and high power objective to examine the pseudohyphae and budding yeast cells (Milne, 2007)

Germ tube production

Germ tube formation was first reported by Reynolds and Braude and hence the germ tube test is also known as Reynolds-Braude Phenomenon. This is a rapid method for identifying and differentiating *C. albicans* and *C. dubliniensis* from other *Candida* spp. The yeast suspended in the fresh human serum by using a sterile loop and incubated at 35-37°C for 2-3 hrs. After the incubation, a drop of suspension was placed on a clean microscope slide and a clean cover glass was placed on the suspension and then examine under the microscope using the low power and high power objective to confirm the presence or absence of germ tubes (Hupert *et al*, 1975).

Corn meal agar with Tween 80

Corn meal agar (HiMedia-146) with tween-80 was used for the demonstration of chlamydospores, blastospores, and pseudohyphae. A suspected colony was picked from SDA using a straight wire and made a deep cut in the corn meal agar plate. The procedure was repeated for each colony. A flamed sterile coverslip was placed over the line of inoculum. The plate was incubated at 22-26°C in the dark for up to 3 days. After the incubation, the plate was examined by placing the plate without its lid on the microscope stage and using a low and high power magnification for chlamydospores, blastospores, and pseudohyphae (Conant *et al*, 1971).

Sugar Assimilation test

The carbohydrate assimilation test forms the foundation to identify the yeast at the species level, which measures the ability of yeast to utilize a specific carbohydrate as the sole source of carbon in the presence of oxygen. Yeast extract agar (HiMedia-M456) medium was prepared as per the manufacturer's instructions. A lawn culture of the pre-incubated broth was made on the yeast extract agar plate and the sugar discs *viz.,* Glucose (HiMedia-DD002), Sucrose (HiMedia-DD013), Maltose (HiMedia-DD005), Cellobiose (HiMedia-DD028), Melibiose (HiMedia-DD030), Inositol (HiMedia-DD027), Trehalose (HiMedia-DD031) and Xylose (HiMedia-DD014) were placed and incubated for 24-72 hrs (Veena *et al*, 2012).

Sugar Fermentation test

Carbohydrate fermentation test detects the ability of certain yeast to ferment a specific carbohydrate. The broth containing 2% Glucose, Sucrose, Lactose, Maltose and Galactose separately with 1% peptone and 0.5% sodium chloride with phenol red indicator (0.005%). Durham's tube was immersed for gas detection. Yeast cultures were inoculated into each carbohydrate broth and incubated at 25°C for one week and examined at 48-72 hours intervals for acid (pink colour) and gas (in Durham's tubes) production (Milne, 2007).

Candida identification kit

Candida identification kit (Himedia-KB006) was used for the identification and differentiation of *Candida* spp. based on twelve conventional biochemical tests. For the preparation of inoculum, a loopful of colonies were taken from SDA medium and inoculated into sterile saline, from the inoculum 50µl were inoculated in each well of kit, then incubated at 25°C for 24-48 hours. Interpretation of results was done as per the standards are given in the identification index.

HICHROME Candida differential agar medium

HiChrome agar is the rapid, plate-based test for the simultaneous isolation and identification of various species of *Candida* which is based on the reactions between the specific

enzymes of the different species and the chromogenic substances. HiChrome agar prepared according to manufacturer's instructions (Himedia-M1456A). 21.02 gm of dehydrated media was suspended in 500 ml of distilled water. The suspension was heated to boil gently (not to be autoclaved) to dissolve the medium completely. After cooling to 50°C, the media were poured into petri plates and allowed to set. The colonies from SDA were sub-cultured on chromogenic medium and incubated at 35°C up to 48 hours to get better developed colonies. The colony morphology (colour, size and texture) were assessed to interpret the identification of species (Vijaya *et al*, 2011 and Agarwal *et al*, 2011).

were recorded as bacteria and 112 (7.96%) were identified as *Candida* spp.

All the 112 isolates were directly subjected to germ tube test to screen the germ tube producers among various *Candida* spp. Out of which, 61 (54.46%) isolates were positive in germ tube production and 51 (45.53%) were negative. Sixty-one germ tube producers were subjected to growth temperature test (growth at 45°C) to differentiate the *Candida dubliniensis* from *Candida albicans* (Table1). Among 61 *Candida* species tested, growth was observed in 52 (85.24%) isolates remaining 9 (14.75%) isolates were failed to growth at 45°C.

Table 1 Differentiation characters between *C. albicans* and *C. dubliniensis*

Candida spp.	Total (n=112)	Germ tube production	Growth at 45°C	Colony morphology	
				Hichrome *Candida* differential agar	Corn meal agar
C. albicans	52 (46.42%)	+	+	Green colonies	yeast+pseudohyphae+Single terminal chlamydospore
C. dubliniensis	9 (8.03%)	+	-	Dark green colonies	yeast+pseudohyphae+multiple terminal chlamydospore

Growth at 45°C

Growth at 45°C has been considered as a suitable test for the differentiation of *C. dubliniensis* (no growth) from *C. albicans* (growth). This test was performed for cultures only those were positive for the germ tube test, chlamydospore production and developed green colonies in CHROM agar. A loopful of the colony was streaked on SDA plate and incubated at 45°C and growth was assessed daily for 10 days (Pinjon *et al*., 1998).

Antifungal susceptibility testing

Antifungal susceptibility testing was performed for all the isolates of *Candida* using disc diffusion method on Mueller-Hinton agar (HiMedia-M173) supplemented with 2% glucose and 0.5 µg / ml of methylene blue. The commercially available antifungal discs were used and zones of inhibition were measured after 24-48 hours at 37°C. The antifungal discs used were Amphotericin-B (100 units), Clotrimazole (10 µg), Fluconazole (10 µg), Itraconazole (30 µg), Ketoconazole (10 µg), Miconazole (30 mcg) and Nystatin (100 units). The zone of inhibition was measured and interpreted as per the instruction manual (HiMedia- *C.albicans* ATCC 90028, *C. glabrata* ATCC 15126, *C. prapsilosis* ATCC 22019, *C. krusei* ATCC 6258 and *C. tropicalis* ATCC 750). *C. albicans* ATCC 90028 was used as quality control strain (CLSI, 2009).

RESULTS

A total of 2, 617 urine samples were collected from the clinically suspected patients. Collected samples were processed, based on a microscopic examination (KOH mount and gram's staining) and colony morphology (MacConkey agar, CLED agar and blood agar) of the organisms; it was classified as bacteria (gram positive bacteria and gram negative bacteria) and fungi (*Candida* and other fungi). In further, suspected isolated colonies (positive for oval budding yeast cells) were subcultured on SDA agar plates for the confirmation of the genus *Candida*. Out of 2, 617 clinical samples, 1, 406 (53.72%) were enrolled as cultures positive and 1, 211 (46.27%) were culture negative. Among culture positive isolates, 1, 294(92.03%) [GPC (n=117) and GNB (n=1177)]

Furthermore, results of biochemical tests (Sugar fermentation, Sugar assimilation and *Candida* identification kit) (Table 2) and colony morphology on (i) Hichrome *Candida* differential agar (green color colonies and produce dark green color colonies respectively) and (ii) corn meal agar medium (yeast+pseudohyphae+single terminal chlamydospore and yeast+pseudohyphae+multiple terminal chlamydospore respectively) were also confirmed this nine strains as *C. dubliniensis*.

Table 2 Biochemical characterization of *C. albicans* and *C. dubliniensis*

Name of the sugars	*C. albicans*	*C. dubliniensis*
Sugar fermentation test		
Glucose	AG	AG
Sucrose	-	-
Maltose	AG	AG
Lactose	-	-
Galactose	V	V
Sugar assimilation test		
Glucose	+	+
Sucrose	+	-
Maltose	+	+
Cellobiose	-	-
Melibiose	-	-
Inositol	-	-
Trihalose	+	-
Xylose	+	-
HiCandida identification kit		
Urease	-	-
Melibiose	-	-
Lactose	-	-
Maltose	+	+
Sucrose	-	-
Galactose	+	+
Cellobiose	-	-
Inositol	-	-
Xylose	+	+/-
Dulcitol	-	-
Raffinose	-	-
Trehalose	+	+

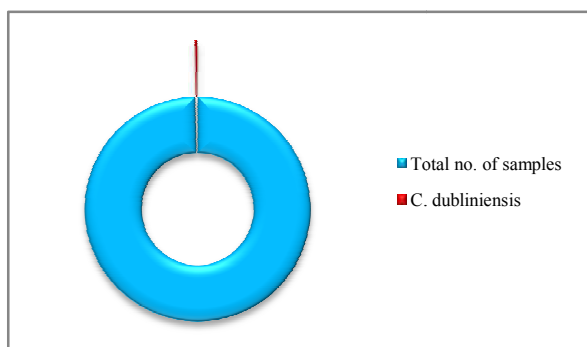In this study overall prevalence *C. dubliniensis* is 0.34% (Fig 1).

Figure 1 Overall prevalence of *C. dubliniensis*

Two classes of antifungal drugs were used for antifungal susceptibility test which were polynes (amphothericin B and nystatin) and azoles (clotrimazole, fluconazole, itraconazole, ketoconazole and miconazole). Antifungal profile of *C. dubliniensis* against antifungal drugs was recorded in the (Fig 2). Among 9 *C. dubliniensis*, all the 9 (100%) isolates were showed sensitive to amphotericin B, followed by fluconazole and ketoconazole 8 (88.88%). Higher resistance was observed for the drugs clotrimazole and nystatin 2 (22.22%). Antifungal sensitive/resistance pattern of nine strains of *C. dubliniensis* was presented in table 3. A maximum number of strains [4(44.44%)] were exhibited the following pattern AP^S CC^S FLC^S IT^S KT^S MIC^S NS^S.
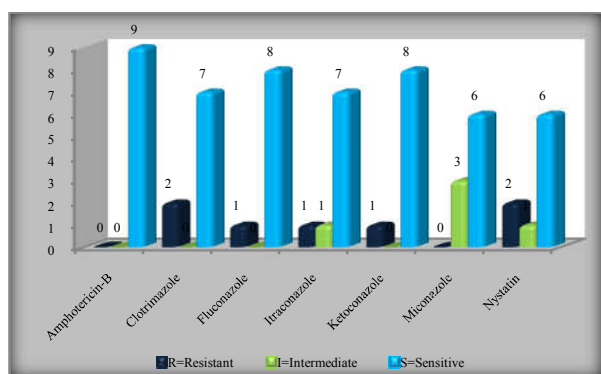

Figure 2 Antifungal profile of *C. dubliniensis*

Table 3 Sensitive/Resistance pattern of *C. dubliniensis*

C. dubliniensis	Antifungal drugs AP/CC/FLC/IT/KT/MIC/NS
n1	S/S/S/S/S/I/R
n2	S/S/S/S/S/S/S
n3	S/S/S/S/S/S/S
n4	S/R/R/I/S/I/S
n5	S/S/S/S/S/S/S
n6	S/S/S/S/S/S/S
n7	S/S/S/S/S/S/S
n8	S/S/S/S/S/S/R
n9	S/R/S/R/I/I/I

DISCUSSION

Candida dubliniensis is considered to be a rising pathogenic yeast. By sharing the phenotypic and genotypic characteristics which are closely related to *C. albicans*, due to this distinctiveness, the identification of this species is still challenging (Guttierrez *et al*, 2002; Sullivan *et al*, 1999). For an example, a retrospective study on yeast stock collections observed approximately 2% of germ tube and chlamydospore

positive isolates were found to be *C. dubliniensis* which were originally identified as *C. albicans* (Coleman *et al*, 1997b; Odds *et al*, 1998). Proper detection of *C. dubliniensis* from the clinical samples is necessary to determine their clinical importance and its role in human disease. The laboratory based differentiation between the *C. albicans* and *C. dubliniensis* remains difficult. On Rice agar, Tween, Tween 80-oxgall-caffeic acid or cornmeal agar *C. dubliniensis* produces chlamydospores more readily and abundantly than *C. albicans* (Sullivan *et al*, 1993; Sullivan and Coleman, 1997; Jabra-Rizk *et al*, 1999a; Koehler *et al*, 1999). On the other hand at 45°C, unlike *C. albicans*, *C. dubliniensis* was unable to grow at the temperature (Coleman *et al*, 1997a; Coleman *et al*, 1997b; Pinjon *et al*, 1998; Sullivan *et al*, 1997b; Sullivan and Coleman, 1998; Jabra-Rizk *et al*, 1999a; Morshhäuser *et al*, 1999). However, a study by Kirkpatrick *et al*. found that 36% of *C. albicans* unable to grow at 45°C (Kirkpatrick *et al*, 1998). Comparably with above mentioned studies, the present study was also used the growth test at 45°C and chlamydospore formation on cornmeal agar for the differentiation of *C. dubliniensis* from the *C. albicans*. A total of 9 (0.34%) isolates were failed to grow at 45°C and from the chalmydospore. The clinical microbiology laboratories stated that *C. dubliniensis* produces much darker green colonies on CHROM agar when compared to *C. albicans* (Schoofs *et al*, 1997). Comparably in the present study, all the isolates of *C. dubliniensis* were produced the dark green color colonies on chromogenic medium.

During the past five years, the documentation of *C. dubliniensis* is increased by improved methods; it has resulted from the existing data about the epidemiology of this species. It is most frequently recovered from the oral cavities of HIV-infected patients (Linares *et al*, 2007), but it has also been isolated from some vaginal samples from HIV-negative and HIV-positive women (Sullivan and Coleman, 1998; Sullivan *et al*, 1995). In HIV patients 6 (7.2%) of isolates were identified as *C. dubliniensis* collected from oropharyngeal swabs (Aher *et al*, 2014). Contrary to an Irish population study, only 3.5% of individuals were carrying *C. dubliniensis* in the oral cavity while in vagina lower prevalence only observed (Ponton *et al*, 2000). The studies from the every continent stated that *C. dubliniensis* has been identified in a wide range of anatomical sites and clinical samples. Likewise, the subsequent authors were reported that the *C. dubliniensis* from different anatomical sites and clinical specimens. In immunocompromised patients, *C. dubliniensis* (1.7%) observed as one of the most frequently isolated species from various clinical samples (Badiee *et al*, 2011). *C. dubliniensis* has also been recovered from blood samples, the neutropenic and solid organ transplantation patients (Gottlieb *et al*, 2001; Meis *et al*, 1999; Sebti *et al*, 2001; Brandt *et al*, 2000). Meanwhile, due to *C. dubliniensis* candidaemia cases were increasing, but epidomiological studies identified, this species rarely and represents a very small proportion of positive blood cultures observed throughout the world (Pfaller and Diekema, 2004; Tortorano *et al*., 2004; Kibbler *et al*, 2003). Polacheck *et al*. described in HIV negative patients five *C. dubliniensis* were isolated, out of which, one strain was recovered from the urine and 4 were recovered from upper respiratory tract and oral specimens (Polacheck *et al*, 2000). Ofonime *et al*. reported that 2 (5.0%) of *Candida dubliniensis* recovered from the lower respiratory

tract infection (Ofonime *et al*, 2013). Uma *et al*. who reported that 31% of *C. dubliniensis* from urine samples of ICU patients (Uma *et al*, 2009), which is very high when compared this study. In this study, 9 (0.34%) of *C. dubliniensis* strains were recovered from the urine specimens of clinically suspected patients. Comparably, a study from Kuwait reported the 2 (0.12%) of *C. dubliniensis* were recovered from the urine samples (Al-Sweih *et al*, 2005).

Though fluconazole resistance *in vitro* has been described in the *C. dubliniensis*, most of the clinical isolates of *C. dubliniensis* were sensitive to existing antifungal agents (Kirkpatrick *et al*, 1998; Moran *et al*, 1998; Moran *et al*, 1997; Pfaller *et al*, 1999; Quindos *et al*, 2000; Ruhnke *et al*, 2000). Similarly Badiee *et al*. noted that no serious problem was observed in the susceptibility pattern of *C. dubliniensis* (Badiee *et al*, 2011). Correspondingly, in the present study, almost all the strains of *C. dubliniensis* were sensitive to antifungal agents. Among seven antifungal drugs used, all the isolates showed sensitive to amphotericin B, followed by fluconazole and ketoconazole (88.88%). Contrarily a study by Aher *et al*. reported that *C. dubliniensis* demonstrated maximum resistance to fluconazole (Aher *et al*, 2014). It may be due to frequent use of fluconazole prophylaxis. Moran *et al*. revealed that overexpression of the major facilitator protein (Mdr1p) mainly responsible for the resistance mechanisms in both clinical and *in vitro*-generated resistant organisms (Moran *et al*, 1998).

CONCLUSION

In conclusion, our study suggested that proper identification of *C. dubliniensis* from different clinical specimens is very essential for the appropriate antifungal therapy. However, the epidemiological data on *Candida* species is increased, but less attention only given to the *C. dubliniensis*. The reason may be due to the problem in phenotypic isolation and identification of this species, which is closely related to *C. albicans*. Many published data concentrate an epidemiology of *C. dubliniensis* particularly in a specific group of populations (HIV and AIDS patients) at specific anatomical sites (Oral cavity), such studies were failed to focus this species in other population. Continuous and proper detection of *C. dubliniensis* are useful for choosing the proper antifungal agents which may reduce the increasing resistance.

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