INCIDENCE AND VIRULENCE TRAITS OF CANDIDA DUBLINIENSIS ISOLATED FROM CLINICALLY SUSPECTED PATIENTS

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ABSTRACT

Objective: Fungal infections are caused by Candida species are increasing mainly in immunocompromised patients. Among Candida species, Candida dubliniensis has recently increasing opportunistic pathogenic yeast. The present study was aimed to assess the incidence and virulence factors of C. dubliniensis isolated from urine samples of various hospitalized clinically suspected patients.

Methods: Totally 1,406 urine samples were processed, among that 9 (0.64%) were identified as C. dubliniensis by germ tube production, biochemical test (Candida identification kit), CHROM agar Candida differential medium and growth at 45°C. Virulence factors of the C. dubliniensis, viz., phospholipase, proteinase, esterase, lipase, hemolysin, and biofilm production were detected by standard procedures.

Results: Maximum number of C. dubliniensis have ability to produce proteinase 6 (66.66%), followed by esterase 5 (55.55%), biofilm formation 4 (44.44%), hemolysin 2 (22.22%), and phospholipase and lipase 1 (11.1%), respectively.

Conclusion: The result revealed that these enzymes are potential virulence factors are the most important thing to realize pathogenesis of C. dubliniensis.

Keywords: Candida dubliniensis, Urine, Incidence, Virulence factors.

INTRODUCTION

Fungal pathogens are becoming increasingly important cause of both community-acquired and nosocomial infections; the genus Candida is the most pathogenic fungi [1]. Although Candida is consisting of more than 200 species, only a few of them has been associated with human infection [2]. Of these, C. albicans is the most important infectious agent and represents a serious public health challenge. In recent years, non-albicans Candida species are also being implicated [3]. In 1995, a new Candida species was identified in human immunodeficiency virus (HIV) infected patient with oropharyngeal candidiasis in Dublin, Ireland [1]. This new species have ability to forms germ tubes and chlamydospores that are almost indistinguishable from those of Candida albicans. Phenotypic characteristics of finding and differentiation of Candida dubliniensis remains problematic in routine identification. On CHROM agar Candida differential medium, C. dubliniensis produced the dark-green colonies within 48 hrs at 37°C when freshly isolated from clinical material, but this property is not retained in subculture [4]. Recently, the absence of growth of C. dubliniensis at 45°C has to be an easy and reliable feature for differentiation of this species from C. albicans [5].

C. dubliniensis has recently been added up to the rising list of potential opportunistic pathogenic yeasts, and it has been recovered from several body sites in many populations; it is most frequently recovered from the oral cavities of HIV-infected patients [6]. Extracellular hydrolytic enzymes seem to play an important role in Candida overgrowth [7]. The enzymes produced are proteinase, lipase, esterase, and phospholipase; factors which are responsible for the invasiveness and proliferation of fungi caused by the destruction of host tissues, in which the organisms are provided with nutrients by the host tissues [8]. Hemolytic activity is another important virulence factor exhibited by the pathogens which allows growth in the host using several iron-binding proteins as a source of iron. In recent years, there has been a rapid spread of antifungal multidrug resistance, which has become a serious public health problem [9]. Biofilm production was also associated with high-level antifungal drug resistance of the associated organisms [10]. Although extensive studies on epidemiology and phylogeny of C. dubliniensis have been performed, little is known about virulence factors such as extracellular enzymes and hemolytic activities [6]. Therefore, the present study was aimed to determine virulence factor (production of proteinase, phospholipase, lipase, esterase, biofilm formation, and hemolytic activity) of C. dubliniensis isolated from urine sample.

MATERIALS AND METHODS

The study included the clinical strains of C. dubliniensis recovered from urine samples of various hospitalized clinically suspected patients from Microbiology Department at Doctors Diagnostic Center, Trichy, India. The collected samples were processed in the Department of Microbiology, Vivekanandha College of Arts and Sciences for Women, Namakkal, Tamil Nadu, India. All the isolates were stored with glycerol on deep freezer until further testing.

Urine sample

A total of 1,406 urine specimens were directly subjected to Gram’s staining in addition specimens were routinely cultured on blood agar, MacConkey, and cystine lactose electrolyte deficient agar (Himedia, Mumbai, India), for the recovery of microbial growth (bacteria and Candida species). Then, the culture plates were incubated at 37°C for 24-48 hrs. After incubation, growth was observed, and suspected colonies
were inoculated on Sabouraud dextrose agar plates supplemented with chloramphenicol (Himedia, Mumbai, India) and incubated first at 37°C for 24-48 hrs to observe the growth of *Candida* spp.

**Mycological investigation**

Then, the isolated colonies were subsequently identified by (i) *Germ* tube production; (ii) conventional biochemical test using Hi *Candida* identification kit (Himedia, Mumbai, India); (iii) subculture of two or three representative colonies on CHROM agar *Candida* differential medium (Himedia, Mumbai, India) and incubation at 37°C for 24-48 hrs. The *Candida* isolates were identified by color characteristics on CHROM agar *Candida* differential medium [11], and (iv) the confirmatory test to differentiate the *C. dubliniensis* from *C. albicans* by growth at 45°C [5].

The temperature test was performed using Sabouraud’s dextrose agar (SDA) with chloramphenicol (Himedia, India). Cultured plates were incubated at 45°C and growth was examined daily up to 10 days. For the tests, a standard reference strain of *C. albicans* (ATCC 90028) was used as the control.

**Phospholipase activity**

*C. dubliniensis* isolates were screened for extracellular phospholipase activity by measuring the size of the zone of precipitation after growth on egg yolk agar [12]. The egg yolk medium consisted of 1.30 g SDA, 1.17 g NaCl, 0.11 g CaCl$_2$ (Himedia, Mumbai, India), and 10% sterile egg yolk (all in 184 ml distilled water). First, these components were mixed and sterilized without the egg yolk; then, the egg yolk was centrifuged at 500 g for 10 minutes at room temperature, and 2 ml of the supernatant was added to the sterilized medium. Standard inocula of the test and control *Candida* isolates (5 ml, with $10^4$ yeast cells [ml saline]$^-$) were inoculated onto the egg yolk agar medium and left to dry at room temperature. Further, 5 ml of saline, but without yeast cells, was overlaid onto the plate and left to dry at room temperature.

Each culture was then incubated at 37°C for 48 hrs after which the diameter of the precipitation zone (Pz) around the colony (an indicator of phospholipase activity) was determined. Reference strains of *C. albicans* (ATCC 90028) served as positive control.

**Proteinase activity**

Proteinase production was measured in terms of bovine serum albumin (BSA) degradation according to the technique described by Stab [1965] [13]. The suspension of $1X10^9$ cells ml$^{-1}$ was prepared from *Candida* isolate. 10 µl of suspension was inoculated on 1% BSA medium plate. The BSA medium consisted of dextrose 2%, KH$_2$PO$_4$ 0.1%, MgSO$_4$ 0.05%, agar 2%, and 1% BSA solution (Himedia, Mumbai, India). The plate was incubated for 5 days at 37°C. After incubation, the plates were fixed with 20% trichloroacetic acid and stained with 1.25% amidoblack. Decolorization was performed with acetic acid. Opaqueness of the agar, corresponding to a zone of proteolysis around the colony was indicative for esterase production by the *Candida* isolate. Reference strains of *C. albicans* (ATCC 90028) served as positive control.

**Biofilm formation**

Biofilm formation was determined for all the isolates, and the standard strains using a method proposed by Branchini et al. (1994) [16]. A loopful of colony from the SDA plate was inoculated into 10 ml of Sabouraud’s liquid medium supplemented with glucose (final concentration of 8%). The tubes were incubated at 37°C for 24 hrs, after which, the broth was aspirated out and the walls of the tubes were stained with safranin. Biofilm formation was scored as negative (0), weak positive (1+), moderate positive (2+), or strong positive (3+).

**Enzymatic score and statistical analysis**

The enzyme activities were expressed as Pz value (a=colony diameter/ b=diameter of the colony plus the precipitation zone) as described by Manns et al. [1994] [17]. The media was prepared by adding 7 ml of aseptically collected fresh sheep blood to 100 ml of SDA supplemented with glucose at a final concentration of 3% (w/v). 10 µl of standard inoculum [10⁴ yeast cells (ml saline)]$^-$ prepared from both the test and the control *Candida* isolates was inoculated onto the medium. The blood agar plate was then incubated at 37°C in 5% CO$_2$ for 48 hrs.

Hemolysin activity was determined by the translucent zone of hemolysis. Reference strains of *C. albicans* (ATCC 90028) served as positive control.

**RESULTS**

A total of 1,406 urine samples were included in the study, among that 9(0.64%) of the strains were identified as *C. dubliniensis* by following characteristics (i) Germ tube production was detected. (ii) as per the HiCandida identification kit result interpretation, *C. dubliniensis* strains were negative in urease activity. Maltose, galactose, xylose,

![Fig. 1: Mycological investigation of Candida dubliniensis (a) Gram-positive budding yeast cells on Gram's staining. (b) Formation of germ tube in human serum at 37°C. (c) Dark-green colonies on CHROM agar medium. (d) Biochemical test results of Candida dubliniensis on HiCandida identification kit](image-url)
and trehalose were utilized. Remaining sugars, viz., melibiose, lactose, sucrose, cellobiose, inositol, dulcitol, and raffinose were not fermented. (iii) dark green colored colonies were observed in CHROM agar Candida differential medium (Fig. 1). (iv) no growth was observed at 45°C.

Virulence factors of C. dubliniensis were demonstrated in Fig. 2. The enzymatic activities of the C. dubliniensis (phospholipase, proteinase, esterase, and lipase) were recorded in Table 1. Out of 9 strains, phospholipase activity was determined in only one strain (11.11%), which showed the moderate activity (Pz=0.5, mean=0.05). Proteinase production was observed in six strains (66.66%). Pz ranged from 0.5 to 0.9 (mean=0.47). Low-level production of esterase was found in five strains (55.55%). Pz ranged from 0.8 to 0.9 (mean=0.47). Lipase activity was noted as one strain (11.11%). Pz ranged from 0.5 to 0.9 (mean=0.10). According to the results of enzyme production, maximum number of strains has an ability to release the proteinase and esterase. Table 2 recorded the results of hemolysin and biofilm production. Beta hemolysis was observed in two strains (22.22%). Biofilm formation was noted in four strains (44.44%).

DISCUSSION

C. dubliniensis is opportunistic yeast, and it appears to have a worldwide distribution [20,21]. 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 [2]. C. dubliniensis have been isolated from a wide range of geographical locations including Europe, North and South America, and Australia [22]. It recovered from several body sites in many populations, but it is most frequently recovered from the oral cavities of HIV-infected patients [6], and 15-30% has been reported from oral cavities of HIV-infected and AIDS patients [23]. Due to the use of inefficient sampling and identification methods or the sampling on the wrong anatomic sites, the prevalence of C. dubliniensis in normal healthy individuals has been underestimated probably [2]. In a study reported that, on an Irish population of normal health individuals, only 3.5% of individuals were found to carry C. dubliniensis in the oral cavity while the prevalence of this species in the vagina was found to be even lower [24] and relatively high prevalence rates of C. dubliniensis have been found in oral cavities of patients suffering from diabetes [22]. As well as being associated with oral infections, C. dubliniensis has also been identified in a wide range of other anatomic sites [2]. Moreover, isolates of C. dubliniensis have been isolated from vaginal, urinary, and fecal specimens [25-27]. Likewise, a study reported that C. dubliniensis (31%) was isolated from urine samples of ICU patients [28]. The percentage of prevalence is very high when compared to our results. In the present study, we identified 9(0.64%) of C. dubliniensis from urine specimens.

Fig. 2: Virulence factors of Candida dubliniensis (a) Proteinase production on bovine serum albumin medium plate, (b) Phospholipase production on egg yolk agar, (c) Lipase production on Sabouraud’s dextrose agar with 1% tributyrin oil, (d) Esterase production on Tween 80 opacity medium (e) Hemolysin production on blood agar plate, and (f) Biofilm formation on Sabouraud’s liquid medium with glucose

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme variables</th>
<th>Percentage</th>
<th>Range (Pz)</th>
<th>Mean±SEM (Pz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. dubliniensis (n=9)</td>
<td>Phospholipase</td>
<td>11.11 (1/9)</td>
<td>0.5</td>
<td>0.05±0.05</td>
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<td></td>
<td>Proteinase</td>
<td>66.66 (6/9)</td>
<td>0.5-0.9</td>
<td>0.47±0.13</td>
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<tr>
<td></td>
<td>Esterase</td>
<td>55.55 (5/9)</td>
<td>0.8-0.9</td>
<td>0.47±0.15</td>
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<tr>
<td></td>
<td>Lipase</td>
<td>11.11 (1/9)</td>
<td>0.9</td>
<td>0.10±0.10</td>
</tr>
</tbody>
</table>

Table 1: Percentage production of enzymatic activities in C. dubliniensis

SEM: Standard error of mean; Pz: Precipitation zone, C. dubliniensis: Candida dubliniensis
Similarly, we use the Tween 80 opacity medium for the detection and 55.55% of esterase activity was determined in C. dubliniensis (Table 1), which is quite high when compared to 16.6% [39]. Lipase activity of Candida plays a key role in persistence and virulence in humans [40]. Lipase is a family of enzymes which hydrolyze the ester bonds in triacylglycerol to yield free fatty acids [41]. In the broad sense of the term, an enzyme which hydrolyzes monoacylglycerol with a long-chain fatty acid (C12 or more) is classified as a monoacylglycerol lipase [36]. C. albicans showed higher lipase activity in this study [42]. None of the studies recorded the lipase production in C. dubliniensis. In our study, we observed that 11.11% of lipase production in C. dubliniensis and Pz ranged at 0.9 (Table 1).

<table>
<thead>
<tr>
<th>Name of the species</th>
<th>Strains name</th>
<th>Hemolysin</th>
<th>Biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. dubliniensis</td>
<td>(n1)</td>
<td>-</td>
<td>1+</td>
</tr>
<tr>
<td></td>
<td>(n2)</td>
<td>β hemolysis</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(n3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(n4)</td>
<td>-</td>
<td>1+</td>
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<td>(n6)</td>
<td>β hemolysis</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>(n9)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

C. dubliniensis: Candida dubliniensis

Since there is essentially no free iron in the human host, most pathogens acquire this indirectly from commonly available iron containing compounds such as hemoglobin [43]. The enzymes involved in this activity are classified as hemolysins. Studies on the activity of hemolysin in Candida were limited. Linares et al. (2007) [6] found the hemolytic activity of C. dubliniensis. A study observed that 60% of hemolysis activity in C. dubliniensis [32], which was high when compared to our results 22.22% (Table 2). Biofilm production is considered to be a potential virulence factor of Candida species. In our study, 44.44% of C. dubliniensis was found as biofilm producers (Table 2). One of the major characteristics of biofilms production is their increased resistance to antifungal drugs. Induction of ergosterol genes has also been described in C. dubliniensis, where incubation with fluconazole and formation of biofilm was coupled with upregulation of the GdrERG3 and GdrERG25 [44].

CONCLUSION

In conclusion, our study recommends that an incidence and virulence factor of C. dubliniensis from clinical specimens is very important. Although number of epidemiological studies on Candida species, less attention only given to the C. dubliniensis. The reason for this may be phenotypic isolation and identification of this species is problematic, which closely related to C. albicans. The majority of studies observed the C. dubliniensis in a specific group of populations (HIV and AIDS patients) such studies were failed to record this species from normal healthy individuals. Knowledge about the virulence factors of C. dubliniensis is clinically very important. Our study revealed that C. dubliniensis had the maximum level of proteinase, esterase activity, biofilm formation and only minimum level of phospholipase, lipase, and hemolytic activity was observed. In addition, prevalence and virulence factors such as esterase, lipase as well as hemolytic activity in C. dubliniensis are less studied and importance must be given.

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