Microbial Pathogenesis 110 (2017) 73–77

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Exoenzyme activity and possibility identification of *Candida dubliniensis* among *Candida albicans* species isolated from vaginal candidiasis

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ARTICLE INFO

Article history:
Received 9 November 2016
Received in revised form
21 May 2017
Accepted 19 June 2017
Available online 20 June 2017

Keywords:
Vulvovaginal candidiasis
C. albicans
RFLP-PCR
Proteinase
Coagulase
C. dubliniensis

ABSTRACT

Objectives: Vulvovaginal candidiasis (VVC) or vaginal candidiasis is a common fungal infection of the genitals causing inflammation, irritation, itching, and vaginal discharge. Common yeast infections are caused by the yeast species *C. albicans*. However, there are other species of *Candida* such as *C. dubliniensis* which are considered as the causative agents of this infection. Hydrolytic enzymes such as proteinase and coagulase are known as virulence factors. The aim of this study was the molecular confirmation and differentiation of *C. dubliniensis* among *C. albicans* strains isolated from women with vulvovaginal candidiasis by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) and the evaluation of proteinase and coagulase activities.

Methods: A total of 100 *C. albicans* strains isolated from women with vulvovaginal candidiasis referred to Shiraz medical clinics were enrolled in the study. All the isolates were primarily identified by conventional methods. PCR-RFLP method was used for the confirmation and identification of *C. albicans* and *C. dubliniensis*. Moreover, *in vitro* proteinase and coagulase activities of these isolates were evaluated using bovine serum albumin media and classical rabbit plasma tube test.

Results: As a result, PCR-RFLP identified 100% of the isolates as *C. albicans*, and no *C. dubliniensis* could be identified in this study. 84% of the isolates showed proteinase activity, whereas coagulase activity was only detected in 5% of the isolates.

Conclusions: This study reveals that *C. dubliniensis* plays no role in vaginal candidiasis in Iranian patients. Proteinase production could be an essential virulence factor in *C. albicans* pathogenicity, but coagulase activity has less potential in this matter.

1. Introduction

Candida spp. are microorganisms that live commensally in different sites of healthy individuals and are considered as a major cause of opportunistic fungal infection in humans [1]. In recent decades, despite advances in healthcare and therapeutic procedures, the incidence of candidiasis has increased dramatically [2,3]. *Candida* spp. is related to infections ranging from superficial or mucocutaneous candidiasis to systemic diseases. However, in the past decades, the incidence of infections caused by non-*C. albicans Candida* (NCAC) species has increased significantly [3]. Vulvovaginal Candidiasis (VVC) or vaginal Candidiasis is a common fungal infection of the genitals [4]. The most common symptoms of VVC include pruritus vulvae, dyspareunia, external dysuria, vaginal soreness, dyspareunia, inflammation, irritation, itching, and vaginal discharge [5]. Common yeast infections are caused by *C. albicans*. This disease occurs in approximately 75% of all adult women at least once during their lifetime [6,7]. 80–92% of cases are caused by

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http://dx.doi.org/10.1016/j.micpath.2017.06.024
0882-4010/

C. albicans. However, other species of *Candida* such as *C. glabrata*, *C. krusei, C. tropicalis*, *C. parapsilosis*, *S. cerevisiae,* and *Trichosporon* spp. are also known as the causes of this infection [8,9].

C. albicans is still dominantly isolated from candidiasis [10]. *Candida dubliniensis* is a new *Candida* species that is closely related to *C. albicans* with phenotypic characteristics [11,12]. Discrimination between *C. albicans* and *C. dubliniensis* is often difficult in clinical samples [12,13]. *C. dubliniensis* is commonly isolated from patients with HIV infection, but occasionally isolated from clinical samples of female genital tract as well. Today, molecular methods such as PCR fingerprinting, ribosomal DNA analysis, and analytical chemistry methods such as Gas-Liquid Chromatography are used to discriminate between *C. dubliniensis* and *C. albicans* [14,15].

The virulence factors of *C. albicans* that contribute to pathogenesis include the production of adhesion to host cells and surfaces of medical devices, biofilm formation, phenotypic switching, secreted aspartyl proteinases (SAPs), and the production of various exoenzymes [16–18]. Extracellular proteases and coagulase play the main role in damaging the host cell membrane and are considered as important virulence factors [17]. These enzymes are effective in the hyphal invasion of the host cells [19]. Proteinase causes the distortion or digestion of host cell membranes to simplify adhesion and invasion of tissues, develops the infection, and destroys cells. These proteins are active in the host's immune system to prevent or resist against microorganisms [20,21]. Coagulase is a protein enzyme that attaches to plasma fibrinogen and causes the conversion of fibrinogen to fibrin, resulting in the clotting of blood [22]. Variations in coagulase expression in different *Candida* isolates may depend on their pathogenicity [23].

As in our previous study [24], RFLP method with two restriction enzyme (*Msp* I and *Bln* I) considered as a reliable method for identification of *C. albicans* and distinguish from *C. dubleniensis,* the purpose of this study was the molecular confirmation and differentiation of *C. dubliniensis* from *C. albicans* species isolated from patients with vaginal candidiasis by PCR-RFLP method in Shiraz (southwest of Iran), and the evaluation of extracellular proteinase and coagulase activities among the isolates.

2. Materials and methods

2.1. Fungal isolates and reference strains

One hundred *C.albicans* strains isolated from woman with vaginal candidiasis were used in this study. These strains were previously identified phenotypically as *C. albicans* on the basis of standard morphologic and physiologic criteria including positive germ tube test (GTT), morphology of chlamydoconidia production on Corn meal agar medium (QLAB, United Kingdom), and green colony color on CHROMagar *Candida* medium (HiMedia, Mumbai, India).

2.2. Molecular identification

2.2.1. DNA extraction from Candida isolates

DNA extraction from the isolates was performed as described by Galan et al. [25]. To this aim, all *C. albicans* isolates were grown on YPD agar at 30 °C for 48 h. Fresh *C. albicans* colonies were collected by a sterile inoculation loop. These colonies were suspended in 1 mL of sterile distilled water to achieve a suspension containing 2×10^6 cells. The tube was centrifuged and the cell fungal pellet was collected. 100 μL of lysis buffer (100 mM Tris–HCl, 0.5% SDS, and 30 mM EDTA) was added to fungal pellet and boiled at 100 °C for 15 min. For DNA purification, 100 mL of the solution of 2.5 M potassium acetate (Sigma, Germany; pH 5.3) was added to the lysis buffer and kept at 4 °C for 1 h. Then, the tube was centrifuged at 12,000 rpm for 5 min. The supernatant was moved to a sterile tube. For the precipitation of DNA, an equal volume of isopropanol was added and mixed well. The samples were centrifuged immediately at 10,000 rpm for 15 min at 4 °C. The supernatant was discarded carefully. The DNA pellet was washed with 200 μL of room-temperature 99% ethanol. The tube was centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was carefully removed without disturbing the DNA pellet. The DNA pellet was air-dried for 20 min and suspended in 50 μL of distilled water. In some isolates, DNA was also purified by tissue DNA extraction mini kit (YTA, Favorgen, Taiwan). The quality of the extracts was measured by spectrophotometric measurement of A_{260} and A_{280} and electrophoresis. 3 μL of extracted DNA from each *Candida* sample was fractionated in 2.2 M formaldehyde and 1.2% Agarose gel at 100 V for 20 min, and then stained using Sybr Green I (Invitrogen, USA) and visualized under ultra-violet light. Afterwards, the extracted DNA was stored in TE buffer at −20 °C [26,27].

2.2.2. PCR-RFLP method

PCR amplification was performed based on Bakshi et al. in a 0.2 mL micro reaction tube using the Thermal Cycler instrument (Analytik Jena, Germany) [28]. 5 μL of extracted DNA was amplified in a final volume of 100 μL with 25 μL of Master Mix, 1 μL of each primer, and 69 μL of distilled water. The pair of universal primers ITS1 (5- TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCC GCTTATTGATATGC-3) was used as forward and reverse primers. The thermal cycler program was adjusted for initial denaturation at 94 °C for 3 min, which preceded a three-stage temperature profile for 30 s at 94 °C as denaturation, 30 s at 51 °C as annealing, and 30 s at 72 °C as extension for 35 cycles, and the final extension for 5 min at 72 °C. We used a negative control without DNA template. The samples were kept at 4 °C in a thermal cycler machine. The amplified products were analyzed using 1.2% (W/V) agarose gel in TBE buffer at 120 v for 15 min, stained using Sybr Green I, and then visualized under ultra-violet light. In the present study, the restriction enzyme *Msp* I (*HpaII*) (Fermentas, USA) was utilized for the identification of *C. albicans* isolates, and the restriction enzyme *Bln* I (*Avr* II) (Fermentas, USA) was used for the differentiation between *C. dubliniensis* and *C. albicans*. The reaction mixture and restriction enzymes *Msp* I (1 μL) and *Bln I* (0.4 μL) were prepared and the experiments were carried out at room temperature in the final volume of 30 μL. The microtubes were incubated at 37 °C for 3 h. Restriction fragments were separated on 2% agarose gel through electrophoresis for 40 min at 90 V. The gel was stained with Sybr Green I and visualized under ultra-violet light [29]. The *Msp* I enzyme produced 2 segments for *C. albicans* species and, after digestion, produced 238 and 297 bp fragment bands [30]. *C. albicans* and *C. dubliniensis* demonstrated a similar pattern with this enzyme. Thus, *Bln I* enzyme was used for the discrimination of these two species; *C. dubliniensis* produced two bands of 355 and 200 bp, while *C. albicans* produced only one fragment of 535 bp [31].

2.3. Determination of proteinase activity

Proteinase production of the isolates was assessed using the plate method described by Akcaglar et al. [32]. Briefly, the isolates were cultured in YEPD medium (2% glucose, 2% bacto-peptone, and 1% yeast extract) at 37 °C for 24 h. A medium was prepared containing 1.17% yeast carbon base, 0.01% yeast extract, and 0.2% Bovine serum albumin (BSA) (all Sigma-Aldrich, USA). When the pH of the medium was adjusted to 5.0, the sterilization was done by filtration, and then the prepared medium was added to a stock solution of autoclaved agar. A suspension containing 1×10^7 cells/mL of fresh colonies was prepared. Filter paper disks (with a