


Candida* Mastitis in Dairy Cattle with Molecular Detection of *Candida albicans

Ibrahim ELDESOUKY ¹  Nayel MOHAMED ² Doda KHALAF ³ Akram SALAMA ²
Ahmed ELSIFY ² Rabee OMBARAK ⁴ Salah EL-BALLAL ⁵ Mohamed EFFAT ³
Mona AL SHABRAWY ³

¹ Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Kafrelsheikh University 33516, EGYPT

² Department of Animal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, University of Sadat City 32897, EGYPT

³ Department of Microbiology and Immunology, National Research Centre, Dokki, Giza 12611, EGYPT

⁴ Food hygiene and control department, Faculty of veterinary medicine, University of Sadat City 32897, EGYPT

⁵ Department of Pathology, Faculty of Veterinary Medicine, University of Sadat City 32897, EGYPT

Article Code: KVFD-2015-14843 Received: 10.12.2015 Accepted: 10.02.2016 Published Online: 11.02.2016

Abstract

This study focused on determining the prevalence of *Candida* species involved in dairy cattle mastitis with molecular detection of *Candida albicans*. A total of 150 milk samples were collected from dairy cattle showing clinical mastitis. Isolation and identification of organisms through phenotypical and physiological criteria on different media were performed as well as molecular identification of *C. albicans*. Forty one isolates of *Candida* species were recovered with a prevalence of 27.3%. *C. albicans* was the dominating species (29.3%). Out of 12 strains phenotypically identified as *C. albicans*, 8 were confirmed by PCR using species specific primer for the 26S rRNA gene of *C. albicans*. A specific virulence determinant Phospholipase B1 gene was detected in all molecularly identified *C. albicans* isolates. This study has clearly shown the prevalence of *Candida* mastitis and providing a new attractive diagnostic molecular tool for mycotic mastitis caused by *C. albicans*.

Keywords: *Candida*, Cattle, Mastitis

***Candida albicans*'ın Moleküler Tespiti ile Sütçü İneklerde *Candida* Mastitisi**

Özet

Bu çalışmada *Candida albicans*'ın moleküler yöntemle belirlenmesi suretiyle sütçü inek mastitlerinde *Candida* türlerinin prevalansının belirlenmesi amaçlanmıştır. Klinik olarak mastitis semptomları gösteren toplam 150 adet sütçü ineğe ait süt örnekleri toplandı. *C. albicans*'ın moleküler yöntemle belirlenmesinin yanında organizmaların fenotipik ve değişik kültürlerdeki fizyolojik kriterleri baz alınarak izolasyon ve identifikasyonu yapıldı. *Candida* türlerinin 41 izolatı %27.3 prevalans oranı ile elde edildi. *C. albicans* en çok belirlenen tür olarak kaydedildi (%29.3). Fenotipik olarak *C. albicans* olduğu belirlenen 12 adetten 8'i 26S rRNA geni için spesifik primer çifti kullanılarak yapılan PCR ile onaylandı. Moleküler yöntemle *C. albicans* olduğu belirlenen tüm izolatlarda spesifik virulans olarak Phospholipase B1 geni tespit edildi. Bu çalışma ile *Candida* mastitislerinin prevalansı belirlenmiş ve *C. albicans* kaynaklı mikotik mastitislerde diagnostik moleküler yöntemin kullanılabilirliği gösterilmiştir.

Anahtar sözcükler: *Candida*, Sığır, Mastitis

INTRODUCTION

Cattle mastitis is regarded as the most prevalent and economically important disease on all continents, with annual great losses in the dairy industry world-

wide ^[1]. A wide variety of microorganisms have been found as etiological agents of mastitis in cattle. In addition to bacterial agents, several other groups of microorganisms such as fungi and algae from *Prototheca* genus capable of inducing an inflammatory process in



İletişim (Correspondence)



+81 85 2306487; Fax: +81 58 2306489



ibrahim543@yahoo.com

the udder^[2]. Yeasts are groups of unicellular opportunistic organisms, ever present in the natural surroundings of dairy cattle and are normal inhabitants of the skin of the udder and teats, in which they exist in low numbers^[3].

Fungal mastitis has been described as related to treatment directed against other pathogens using contaminated syringes, cannulas, or contaminated antibiotic preparations^[4]. In recent years, predominance of the genus *Candida* was reported in various studies of cattle mastitis^[5,6]. Several species of *Candida* have been implicated in subclinical and clinical mastitis^[6]. Identification of *Candida albicans* by conventional methods based on morphological features and reproductive structures may take days to weeks to develop in culture, and evaluation of these characteristics requires expertise in mycology^[7]. PCR-based detection of fungal DNA sequences can be rapid, sensitive and specific^[8]. Phospholipase B1 (PLB1) considers one of the main virulence factor secreted by *C. albicans*. This enzyme digests the phospholipid constituents of host cell membrane leading to cell lysis with alterations of surface characteristics that facilitate adherence and subsequent infection^[9]. Few reports exist on the prevalence of fungal mastitis in Egyptian cattle specially those caused by *Candida* species. Therefore, the aim of the present study was to determine the prevalence of *Candida* species in dairy cattle suffering from mastitis, with molecular based identification of *C. albicans* isolates.

MATERIAL and METHODS

Sampling, Isolation And Identification Procedures

A total of 150 milk samples were collected from dairy cattle showing clinical mastitis from 3 different dairy cattle farms at Minufiya Governorate, Egypt. The study was complied with the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, University of Sadat city. Milk samples (100 µl) were inoculated in Sabouraud Dextrose Broth and then incubated at 37°C for 24 h. Thereafter, 50 µl of each broth cultures was plated on Sabouraud Dextrose Agar (SDA) with Chloramphenicol (0.05 mg/ml) and chromogenic agar (Pronadisa Laboratorios Conda, S.A.).

The plates were incubated at 37°C for 72 hs. Yeast identification was performed based on the observed morphological characteristics, like formation of Chlamydoconidia, pseudohyphae and germ tube development. Additional characteristics taken into account include; growth at 45°C, growth in the presence of 0.1% cyclohexamide (Sigma TM), urea hydrolysis, acidic pH tolerance, and carbohydrates assimilation and/or fermentation (glucose, galactose, lactose, maltose, xylose, and sucrose), accordingly to the methodology described by Barnett et al.^[10].

Molecular Identification of *C. albicans*

Total chromosomal DNA was isolated from *C. albicans* (Analytik jena kit, according to the manufacturer's instructions) and subjected to PCR amplification. The primer set was previously designed as species universal primer specific for the amplification of a fragment of 175 bp specific to the 26S rRNA of *C. albicans*^[11]. DNA samples were amplified in a total of 25 µl of the following reaction mixture: 12.5 µl DreamTaq TM Green Master Mix (2X) (Thermo Fisher Scientific, Inc), 1 µl of each primer (10 µM) (Biobasic inc. company, Canada), 5 µl template DNA (1 µg) and 5.5 µl nuclease-free water. Amplification was carried out in the thermal cycler according to the following protocol: initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. The amplified PCR product was electrophoresed on a 1.5% agarose gel in Tris-Acetate-EDTA buffer. A 100-bp DNA ladder (Invitrogen, Carlsbad, CA) was used as a molecular weight marker.

Detection of the Phospholipase B1 in *C. albicans* Isolates

Total chromosomal DNA isolated from *C. albicans* culture was subjected to PCR with oligonucleotide primers previously designed by Mukherjee et al.^[12]. PCR amplification reaction mixtures was performed in a final volume of 25 µl consisted of 12.5 µl DreamTaq TM Green Master Mix (2X) (Thermo Fisher Scientific, Inc), 1 µl of each primer (10 µM) (Biobasic inc. company, Canada), 5.5 µl nuclease free water and 5 µl template DNA (1 µg). The PCR cycling conditions consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 47°C for 1 min, and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. The amplified PCR product was electrophoresed on a 1.5% agarose gel in Tris-Acetate-EDTA buffer.

RESULTS

Prevalence of *Candida* Species in Dairy Cattle with Mastitis

Out of 150 milk samples analyzed from cattle with mastitis, 41 *Candida* isolates were recovered (27.3%). Based on cultural and morphological and biochemical characteristics, six different species of *Candida* were identified. *C. albicans* was the predominant one (29.3%), followed by *C. tropicalis*, *C. guilliermondii* (19.5%, each), *C. glabrata* (14.6%), *C. krusei* (12.2%) and *C. kefyr* (4.9%) (Table 1).

Molecular Identification of *C. albicans*

In this study, PCR based on species specific universal oligonucleotides primers was used to confirm phenotypic identification of *C. albicans*. Out of 12 isolates presumptively

Table 1. *Candida* species isolated from cattle with mastitis
Tablo 1. Mastitisli ineklerden izole edilen *Candida* türleri

Candida Isolates	No	%
<i>C. albicans</i>	12	29.3
<i>C. tropicalis</i>	8	19.5
<i>C. guilliermondii</i>	8	19.5
<i>C. glabrata</i>	6	14.6
<i>C. krusei</i>	5	12.2
<i>C. kefyr</i>	2	4.9
Total	41	100

high incidence of mycotic mastitis especially that caused by *Candida* species has been noticed recently [4]. In the present study, the percentage of *Candida* isolation was 27.3%. Similar results were obtained by Zaragoza et al. [13] in Mexico (25.7%) and Geraldo et al. [14] in Brazil (29.35%). However, many studies conducted in other countries revealed higher frequency of *Candida* mastitis (79.4% in China and 71.9% in Algeria) respectively [5,6]. A higher percentage of isolation of *Candida* species from clinical cases reveal that the incidence of yeast mastitis is increasing which may be due to unhygienic conditions. Moreover, the development of antibiotic resistance in

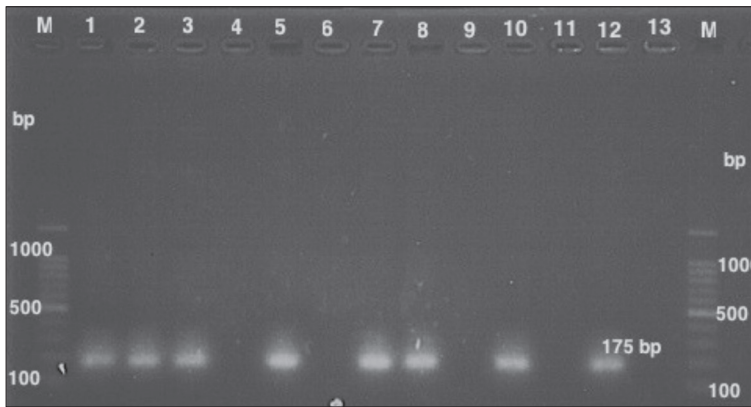
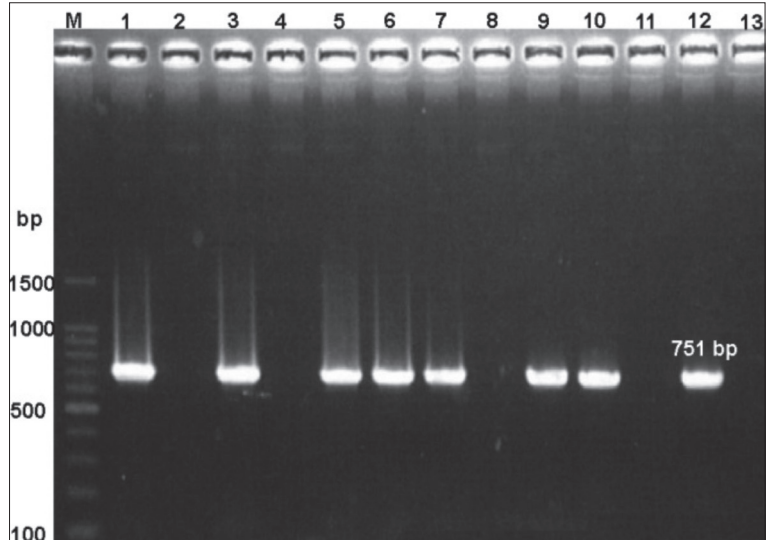


Fig 1. PCR test for detection of *C. albicans*. Agarose gel electrophoresis of amplified PCR products (175bp) by using species specific primer of *C. albicans*. Lane M: 100 bp ladders molecular size marker, lanes 1-3, 5, 7-8, 10 and 12 represent *C. albicans*, lanes 4, 6, 9 and 11 represent non *C. albicans* isolates, lane 13 control negative

Şekil 1. PCR ile *C. albicans*'ın belirlenmesi. *C. albicans* için tür spesifik primerler kullanarak PCR ürünlerinin (175bp) amplifikasyonu ve agaroz jel elektroforeziste gösterilmesi. M: 100 bp moleküler uzunluk markırı, 1-3, 5, 7-8, 10 ve 12: *C. albicans* pozitif örnekler, 4, 6, 9 ve 11: *C. albicans* negatif örnekler, 13: negatif kontrol

Fig 2. PCR test for detection of Phospholipase B1 in *C. albicans* isolates. Agarose gel electrophoresis of amplified PCR products (751bp) by using specific primer of Phospholipase B1 gene. Lane M: 100 bp ladders molecular size marker, lanes 1, 3, 5-7, 9-10 and 12 represent *C. albicans* isolates positive for Phospholipase B1. Lanes 2, 4, 8 and 11 represent non *C. albicans*. Lane 13 control negative

Şekil 2. PCR ile *C. albicans* izolatlarında Phospholipase B1'in belirlenmesi. Phospholipase B1 genine spesifik primerler kullanarak PCR ürünlerinin (751bp) amplifikasyonu ve agaroz jel elektroforeziste gösterilmesi. M: 100 bp moleküler uzunluk markırı, 1, 3, 5-7, 9-10 ve 12: Phospholipase B1 pozitif *C. albicans* izolatları, 2, 4, 8 ve 11: *C. albicans* negatif, 13: negatif kontrol



identified by phenotypic methods as *C. albicans*, 8 isolates yield the predicted 175 bp DNA fragments, while the other four isolates were negative (Fig. 1). Specific PCR was carried out on molecularly identified *C. albicans* species to detect Phospholipase B1 gene as a virulence determinant. The PCR produced a DNA fragment of predicted size (751 bp) in all molecularly identified *C. albicans* isolates (Fig. 2).

DISCUSSION

The prevalence of mastitis related to yeasts is usually low as compared with other agents of mastitis. However,

the bacteria, which prolongs the course of treatment favoring chances for the fungal species such as *Candida* to infect as secondary invader. The present study showed a clear overall predominance of *C. albicans* among the *Candida* species. This finding confirms a tendency seen in another study conducted in India [15], but contrary the other report that described low prevalence of *C. albicans* in mastitic cattle [5]. The present study revealed that *C. tropicalis* and *C. guilliermondii* were the second most frequent species isolated while *C. krusei*, and *C. kefyr* were less frequently isolated, in contrast to some reports [5,6].

It is known that the phenotypic characterization of yeasts can lead to errors due to the fact that several species present similarities in their morphologies and biochemical/physiological characteristics [16]. Recently, molecular biology-based techniques have been adapted to the identification of *C. albicans* which include DNA-DNA tests using analyses with restriction endonucleases, methods based in pulsed field gel electrophoresis, DNA tests using probes. In particular, PCR has increasingly been used for *Candida* diagnosis, as it is quick, simple, specific, sensitive and reliable [11]. In the present study, 12 isolates identified morphologically as *C. albicans* were tested using species universal primer amplifying a fragment of the rRNA gene (175 bp) that could distinguish individual *C. albicans* from other *Candida* species. Out of 12 *C. albicans* isolates, 8 isolates yield DNA fragments of the predicted size. The PCR negative strain could be *C. dubliniensis* as the phenotypic methods for the identification of *Candida* species are often unable to discriminate *C. albicans* and *C. dubliniensis* [17]. *C. albicans* Phospholipase B1 gene is considered important virulence determinant, and could potentially facilitates increased penetration of fungal hyphal elements by directly damaging host cell membranes [9]. PCR was used by many authors for detection of phospholipase activity in pathogenic fungi [18]. In the current study, all isolates of genetically identified *C. albicans* were tested and identified with specific primers identical to the 751-bp region of the Phospholipase B1 gene.

The results of the present study reveal the relatively higher incidence of *Candida* mastitis especially *C. albicans* which seems to be of interest as a probable cause of mycotic mastitis in dairy cattle. It is evident that reliance on the variable expression of phenotypic characteristics of isolated yeasts can lead to inconsistent results. Phospholipase B1 provides new attractive and diagnostic targets for mycotic mastitis caused by *C. albicans*.

REFERENCES

- Bradley A:** Bovine mastitis: An evolving disease. *Vet J*, 164, 116-128, 2002. DOI: 10.1053/tvjl.2002.0724
- Krukowski H, Lisowski A, Rozanski P, Skorka A:** Yeasts and algae isolated from cows with mastitis in the south-eastern part of Poland. *Pol J Vet Sci*, 9, 181-184, 2006.
- de Casia dos Santos R, Marin JM:** Isolation of *Candida* spp. from mastitic bovine milk in Brazil. *Mycopathologia*, 159, 251-253, 2005. DOI: 10.1007/s11046-004-2229-2
- Dworecka-Kaszak B, Krutkiewicz A, Szopa D, Kleczkowski M, Bieganska M:** High prevalence of *Candida* yeast in milk samples from cows suffering from mastitis in Poland. *Sci World J*, 2012, Article ID: 196347, 2012. DOI: 10.1100/2012/196347
- Zhou Y, Ren Y, Fan C, Shao H, Zhang Z, Mao W, Wei C, Ni H, Zhu Z, Hou X, Piao F, Cui Y:** Survey of mycotic mastitis in dairy cows from Heilongjiang Province, China. *Trop Anim Health Prod*, 45, 1709-1714, 2013. DOI: 10.1007/s11250-013-0419-y
- Ksouri S, Djebir S, Hadeif Y, Benakhla A:** Survey of bovine mycotic mastitis in different mammary gland statuses in two north-eastern regions of Algeria. *Mycopathologia*, 179, 327-331, 2015. DOI: 10.1007/s11046-014-9845-2
- Pfaller M, Wenzel R:** Impact of the changing epidemiology of fungal infections in the 1990s. *Eur J Clin Microbiol Infect Dis*, 11, 287-291, 1992. DOI: 10.1007/BF01962067
- Makimura K, Murayama SY, Yamaguchi H:** Detection of a wide range of medically important fungi by the polymerase chain reaction. *J Med Microbiol*, 40, 358-364, 1994. DOI: 10.1099/00222615-40-5-358
- Ibrahim AS, Mirbod F, Filler SG, Banno Y, Cole GT, Kitajima Y, Edwards Jr JE, Nozawa Y, Ghannoum MA:** Evidence implicating phospholipase as a virulence factor of *Candida albicans*. *Infect Immun*, 63, 1993-1998, 1995.
- Barnett JA, Payne, RW, Yarrow D:** Yeast, Characteristics and Identification. Third ed., Cambridge University Press, Cambridge, UK, 2000.
- Mannarelli BM, Kurtzman CP:** Rapid identification of *Candida albicans* and other human pathogenic yeasts by using short oligonucleotides in a PCR. *J Clin Microbiol*, 36, 1634-1641, 1998.
- Mukherjee PK, Seshan KR, Leidich SD, Chandra J, Cole GT, Ghannoum MA:** Reintroduction of the PLB1 gene into *Candida albicans* restores virulence *in vivo*. *Microbiol*, 147, 2585-2597, 2001. DOI: 10.1099/00221287-147-9-2585
- Zaragoza CS, Olivares RA, Watty AE, Moctezuma Ade L, Tanaca LV:** Yeasts isolation from bovine mammary glands under different mastitis status in the Mexican High Plateau. *Rev Iberoam Micol*, 28, 79-82, 2011. DOI: 10.1016/j.riam.2011.01.002
- Costa GM, Pereira UP, Souza-Dias MA, Silva N:** Yeast mastitis outbreak in a Brazilian dairy herd. *Braz J Vet Res Anim Sci*, 49, 239-243, 2012.
- Pachauri S, Varshney P, Dash SK, Gupta MK:** Involvement of fungal species in bovine mastitis in and around Mathura, India. *Vet World*, 6, 393-395, 2013. DOI: 10.5455/vetworld.2013.393-395
- Spanemberg A, Ramos JP, Leoncini O, Alves SH, Valente P:** High frequency of potentially pathogenic yeast species in goat's raw milk and creamed cheese in Southern Brazil. *Acta Sci Vet*, 37, 133-141, 2009.
- Marinho SA, Teixeira AB, Santos OS, Cazanova RF, Ferreira CA, Cherubini K, de Oliveira SD:** Identification of *Candida* spp. by phenotypic tests and PCR. *Braz J Microbiol*, 41, 286-294, 2010. DOI: 10.1590/S1517-83822010000200004
- Ghannoum MA:** Extracellular phospholipases as universal virulence factor in pathogenic fungi. *Med Mycol J*, 39, 55-59, 1998.