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Clonal diversity and antifungal susceptibility of Candida spp. recovered from cow milk

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Abstract

The aim of this study was the isolation, identification, phylogenetic analysis and antifungal susceptibility of *Candida* spp. from milk samples of healthy and mastitic cows in Kayseri/Turkey. Milk samples from 300 cows were found to be negative/positive for mastitis with the California Mastitis Test. *Candida* spp. was isolated by using the Brillance *Candida* Agar Base. Phenotypic tests, Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF), and VITEK-2 analyses were applied to confirm the obtained isolates. Resistance to flucytosine, fluconazole and caspofungin antifungals of the isolates were determined by Etest and VITEK-2. The genetic homologies of *Candida* spp. isolates were determined by Repetitive Extragenic Palindromic-PCR (RepPCR). In this study 62 (from 53 healthy, 9 mastitic) yeast isolates were obtained and 37 (59.6 %) were identified as non-albicans *Candida* (NAC) species. Phenotypic tests revealed that out of 62 isolates, 29 (46.7 %), 4 (6.5 %), 3 (4.8 %), 1 (1.6 %) and, 24 (38.7 %) were identified as *Candida lusitaniae, Candida catenulate, Candida tropicalis, Candida silvicola* and other yeast species, respectively. Only one sample (1.6 %) was identified as *Candida albicans* by MALDI-TOF however, according to VITEK-2, the agent was not confirmed as *C. albicans*. According to antifungal susceptibility testing by VITEK-2, one (2.7 %) of the isolates was resistant to fluconazole, one (2.7 %) was resistant to caspofungin, and 4 (10.8 %) were resistant to flucytosine. However, using E test, 10 isolates (27 %) were resistant to flucytosine. Using Rep-PCR, eight genotypic clones were observed. Genotype F (13.8 %) and G (13.8 %; 2 subtypes) were common clones in this study. In conclusion, NAC species were detected in healthy and mastitic cow milk samples. Epidemiological studies need to be conducted to track effectively the main source and to understand the diversity and distribution of the agent. It is necessary to consider the potential risks of yeast contamination in milk for public health. It is essential to focus on adequate sanitation procedures and storage conditions of milk.

Key words: **antifungal resistance, MALDI-TOF, VITEK-2, molecular typing, milk, non-albicans *Candida* (NAC) species**

Introduction

Candida spp. are opportunistic fungal pathogens that are mostly found in the natural surroundings of cows and live commensally in the digestive system, oral cavity and vagina, however it can also cause endogenous infections especially in immunocompromised and hospitalized individuals (Carter and Wise, 2004; de Casia dos Santos and Marin, 2005). Moreover, yeasts of the Genus *Candida* consist of approximately 200 yeasts (Sullivan et al., 2005) among which *C. albicans*, is the most common (Deorukhkar et al., 2014) although a novel focus has been shifted towards non- albicans *Candida* (NAC) species.

Fungal mastitis in cows causes a high amount of economic losses in the dairy industry (Abou-Elmagd et al., 2011). Most cases of fungal mastitis are attributed to *Candida* spp. which are probably caused by injuries during the use of intramammary antibiotics (Du Preez 2000; Elad et al., 1995; Quinn et al., 2002). Little is known about NAC species such as *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* and *Candida dubliniensis* (Spampinato and Leonardi, 2013; Deak, 2006) as causative agents in the etiology of mycotic mastitis. Previously rarely reported NAC species infections are currently rising (Pfaller et al., 2014).

Several studies have been conducted to assess the accurate identification of *Candida* spp. isolates at the species level. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI TOF-MS) has recently been reported as an efficient method for rapid and correct identification of bacteria as well as yeast, directly from the positive sample, compared with conventional methods (Marinach-Patrice et al., 2010). The MALDI TOF-MS technique is based on the determination of microorganism protein fingerprints (Dhiman et al., 2011). Among the other DNA-based techniques, Repetitive Extragenic Palindromic-PCR (Rep-PCR), is more preferable, for being faster, low-cost, and reproducible in phylogenetic analysis (Abaci, 2009; Saghrouni et al., 2013).

The emergence of antifungal resistant yeast species has represented a significant public health concern. Therefore, the rapid and accurate determination of agent and antifungal susceptibility testing have become necessary to make valid decisions and to guide therapy (Pulcrano et al., 2013).

Literature screening has revealed few reports on the incidence of *Candida* genus as a causative agent of mastitis, which in turn results in a large amount of economic losses in Turkey. In this respect, the aim of this study was to isolate and identify *Candida* spp. from the milk of healthy and mastitic cattle in Kayseri and to determine the antifungal susceptibility and molecular typing of the isolates.

Materials and methods

Sampling

In the present study, a total of 300 milk samples composed of 150 healthy and 150 cattle with mastitis were collected from 5 different dairy farms in Kayseri, Turkey from 2015 to 2016.

Mastitis was identified by clinical signs (in the udder including pain, swelling, warmth, and abnormal appearance of milk). Furthermore, cows without clinical mastitis signs were diagnosed by using a California Mastitis Test (CMT) at each farm. Initially, the nipples were cleaned with 70% ethyl alcohol swabs. After discarding the foremilk approximately 2-5 mL of milk was taken into the CMT paddle, and an equal amount of commercial CMT reagent was added into each cup, while moving the paddle in a gentle circular a few seconds. A total of 1200 quarter CMT reactions were recorded in an ordered scale as either, 0, 1, 2, or 3, with 0 indicating no reaction, 1 being trace and a slight positive, and 2 and 3 as definite positive. The results were scored according to gel formation and color changes to blue-purple (Kasikci et al., 2012). All milk samples were transported on ice to the laboratory immediately after harvest for same day analysis.

Candida spp. isolation

The samples were taken and inoculated in Sabouraud dextrose broth (Merck, Germany). After being incubated at 37 °C for 48-72 h, the broth culture was plated on Chrom agar (Brillance Candida Agar Base, CM1002, Oxoid, UK) with supplement (SR0231E Oxoid, UK), and incubated at 37 °C for 48-72 h under aerobic conditions. Presump-

tive *Candida* spp. colonies were selected according to colony morphology and were inoculated onto blood agar (Blood agar base No:2, Merck, Germany) containing 7 % defibrinated sheep blood and Sabouraud dextrose agar (Merck, Germany).

For phenotypic identification of *Candida* spp. from suspected colonies; Gram staining and microscopic examination, morphological features (germ tube test, chlamydoconidia production test, and germinal tube development), urea hydrolysis, carbohydrate assimilation and /or fermentation tests were utilized. The *Candida albicans* ATCC 10231 reference strain was included as a quality control strain.

MALDI-TOF and VITEK-2 System for identification

All *Candida* spp. isolates were subcultured onto Yeast Extract Agar (Merck, Germany) and incubated at 30 °C for 24 h. MALDI-TOF analysis was performed according to previous studies (Pulcrano et al., 2012; Pulcrano et al., 2013) sampling directly from positive yeast colonies.

Identification and antifungal susceptibilities of isolates by the Vitek 2 system were performed in the Central Laboratory of Erciyes University Medical Faculty using VITEK® 2 YST ID card and AST-YS07 Vitek 2 card, respectively.

Antifungal Susceptibility Test

The E test method was used for the determination of susceptibilities of the isolates as described by NCCLS 2002 (M27-A2) directions. Three antifungal agents were used to evaluate the antifulgal resistance profiles of the isolates including flucytosine, fluconazole, and caspofungin (Oxoid, UK). For each *Candida* isolates; the density was adjusted to McFarland No. 0.5 turbidity (106 cell mL^{-1}) standards, the suspension at 100 µL volume was inoculated onto RPMI 1640 (2 % glucose and 1.5 % agar, MOPS added, Oxoid, UK) agar. The antifungal susceptibility results were evaluated as resistant, intermediate and sensitive.

Rep-PCR DNA fingerprinting

DNA extraction of the isolates were performed by using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) and all DNA amplification was applied by using the DiversiLab Candida kit for DNA fingerprinting (BioMérieux, France) according to the manufacturer's instructions. Briefly, 2 µL of DNA were added to the rep-PCR master mix in a total volume of 25 µL per reaction. The protocol was as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 92 °C for 30 sec, annealing at 50 °C for 30 sec, and extension at 70 °C for 90 seconds, with a final extension at 70 °C for 3 min.

DiversiLab version 2.1.66 web-based interpretation software was used to obtain the gel images and electropherograms (Fig. 1) of the rep-PCR based fingerprint patterns for each the *Candida* isolates. A similarity calculation was carried out by the Pearson correlation coefficient. To compare the rep-PCR profiles automatically; the unweighted pair group method with arithmetic mean (UPGMA) was used.

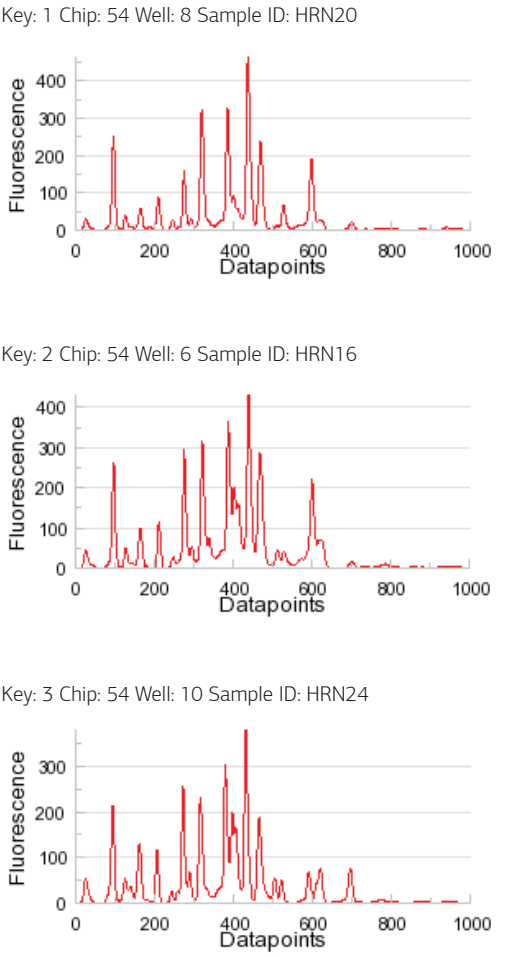

FIGURE 1. Electropherogram samples of *Candida* spp. isolates

Results and discussion

The data on the percentage of *Candida* members among the total fungi isolates in milk ranged from 37.5 % to 100 %, while the rates of *C. albicans* were found to be 8.9 % (de Casia dos Santos and Marin, 2005), 12.5 % (De Soares E Barros et al., 2011), 13.75 % (Gogoi et al., 2014), and 33.34 % (Da Costa et al., 2012).

In this study, 37 *Candida* spp. isolates from the 300 (12.3 %) milk samples were obtained with no isolates of *C. albicans*. This *Candida* spp. rate is relatively high as obtained by Du et al. (2018) from China (23.44 %), compared to that reported from Slovenia as 7.5 % (Pengov, 2002), Brazil as 17.3 % and 12.8 % (de Casia dos Santos and Marin, 2005; Sartori et al., 2014), and in Turkey as 12.7 % and 17.7 % (Seker, 2010; Erbas et al., 2017).

Phenotypic tests revealed 29 (46.7 %), 4 (6.5 %), 3 (4.8 %), 1 (1.6 %), and 24 (38.7 %) of 62 isolates to be NAC species., with the predominant species of *C. lusitaniae* followed by *C. catenulate, C. tropicalis, C. silvicola* and other yeast species, respectively (Table 1). In parallel to our study, Du et al. (2018) obtained nine different NAC species in 60 of 256 mastitic milk samples and reported no *C. albicans*. Although *C. albicans* is considered as the most common and virulent species among the *Candida* spp., recent reports from clinical specimens highlight the increasing rate of *C. lusitaniae* isolates (Ozcan et al., 2016; Zhao et al., 2017)

According to a study conducted by Cilvez (2017), *Candida* spp. was analysed in 24 % (96/400) of the milk samples from which 16.7 % of the isolates were identified as *C. albicans* and 83.3 % were NAC species. *C. krusei* (44.5 %), was the leading species and *C. albicans* was found at a rate of 8.9 % of milk isolates in a study conducted by de Casia dos Santos and Marin (2005) in Brazil. Likewise, some authors have also reported a high frequency of *C. krusei* isolates among the other species (de Casia dos Santos and Marin, 2005; Sartori et al., 2014; Turkyilmaz and Kaynarca, 2010). Currently, about 40 different *Candida* species have been documented in human infections although six *Candida* species (*C. albicans, C. tropicalis, C. glabrata, C. parapsilosis, C. kefyr,* and *C. krusei*) have been shown to be predominant etiological agents of candidiasis (Miceli et al., 2011). In addition, *C. parapsilosis, C. krusei* and other non-albicans species are reported as prominent species to pose mycotic mastitis in cows (Du et al., 2018; Rodrigez et al., 2010).

The variety of NAC isolates obtained from mastitic milks in this study suggest the possible pathogenic role of NAC species in the etiology of subclinical mastitis in this region.

Moreover, *Candida* spp. isolates (53/150) obtained from the healthy milk cows in this study points out improper hygiene and health practices in milk enterprises. Regarding the high determination rates of NAC species in this region, several factors could be of concern including abused use of antibiotics for mastitis treatments, contaminated feed, and improper milking practices.

In this study, antimicrobial resistance was detected only in *C. lusitaniae* isolates. Caspofungin, fluconazole, and flucytosine resistance were observed from 1 (2.7 %), 1 (2.7 %), and 4 (10.8 %) of the isolates with VITEK-2, respectively whereas 10 (27 %) isolates were only resistant to flucytosine according to the E test. However, in a study conducted by Mendes et al., (2018) 5.65 % of the *Candida* species isolates from 27 mastitic milk samples detected had resistance to fluconazole, whereas none of the isolates had flucytosine resistance. Du et al., (2018) reported that all of the *C. krusei* strains isolated from mastitic milk were resistant to fluconazole and flucytosine and all *C. parapsilosis* isolates were susceptible to fluconazole and resistant to flucytosine. Sonmez and Erbas (2017) also reported that all of the *Candida* species isolated from mastitic milk were resistant to fluconazole and flucytosine. Based on the antifungal resistance profiles of the isolates obtained in this study, it is worth to recommending not using these antifungal agents in the treatment of mastitis caused by NAC species in this region.

Previous studies comparing the Vitek-2 system with the E test and conventional methods to determine the antifungal susceptibility of *Candida* species, reported that the ability of the VITEK 2 system to provide quantitative results was more reproducible and accurate (Cretella et al., 2016; Bourgeois et al., 2010; Cuenca-Estrella et al., 2010; Melhem et al., 2014).

The Vitek® MS - Mass spectrometry (bioMérieux) is used for in vitro diagnosis and microbial identification a unique and first American approved and

FDA certified mass spectrometer (Mendes et al., 2018). The Vitek® 2 Compact system is commonly utilized by researchers to identify microorganisms and is a practical and efficient machine (Melhem et al., 2014; Zhang et al., 2014; Mendes et al., 2018).

In the Rep-PCR fingerprinting stage of the study, one of 37 isolates could not be cultured. Therefore, the Rep-PCR protocols were applied on the remaining 36 isolates. In this study, the percentage similarity rates and dendrogram report were generated for each isolate in data analysis. The dendrogram and similarity matrix of *Candida* spp. isolates were shown in Fig. 2 and Fig. 3 respectively. The genotyping of *Candida* strains by rep-PCR based fingerprinting demonstrated the presence of eight major genotypes. Of the 36 *Candida* spp. strains, 5 were shown to belong to the dominant genotype F.

Rep-PCR analysis is a method used for demonstrating clonal relationships. It is easy to perform and has a faster return time compared to other molecular typing methods and it is a rapid method of monitoring *Candida* spp. outbreaks and hence, it permits timely control measures to be taken (Koc et al., 2017).

The present study indicates that the MAL-DI-TOF MS systems are reliable and cost-effective techniques for the identification of NAC species. Identification of NAC would be faster and more precise than that of conventional methods. Moreover, the use of MALDI-TOF MS systems should improve identification rates, the diagnosis of fungal infections, and allowing for appropriate antifungal therapy (Lacroix et al., 2013)

Several studies have been conducted using the DiversiLab system to focus on the differentiation or typing of *Candida* species (Wise et al., 2007; Diab-Elschahawi et al., 2012; Mutlu Sariguzel et al., 2015; Guducuoglu et al., 2016; Zhao et al., 2017).

In this study, percentage similarity rates and dendrogram reports were generated for each isolate in the data analysis. The dendrogram and similarity matrix of *Candida* spp. isolates are shown in Fig 2 and 3. Isolates with a similarity of 90 % or more were classified as the main clones, those with a similarity of 95 % or more as subclones, and strains showing 90% or less were evaluated to be different clones. Briefly, as a result of Rep-PCR, eight clones were observed: A (8.3 %; 2 subtypes), B (11.1 %), C (8.3 %; 2 subtypes), D (5.5 %), E (8.3 %), F (13.8 %), G (13.8 %; 2 subtypes) and H (8.3 %). Clone F (13.8 %) and G (13.8 %; 2 subtypes) were common genotypes in this study.

TABLE 1. Distribution of *Candida* spp. obtained from mastitic and healthy cow milk

	MALDITOF-MS			VITEK-2		
***Candida* species**	**Healthy cow**	**Mastitic cow**	**Total**	**Healthy cow**	**Mastitic cow**	**Total**
C. lusitaniae	25 (40.3 %)	4 (6.5 %)	29 (46.7 %)	25 (40.3 %)	4 (6.5 %)	29 (46.7 %)
C. catenulate	4 (6.5 %)	-	4 (6.5 %)	4 (6.5 %)	-	4 (6.5 %)
C. tropicalis	1 (1.6 %)	2 (3.2 %)	3 (4.8 %)	1 (1.6 %)	2 (3.2 %)	3 (4.8 %)
C. silvicola	1 (1.6 %)	-	1 (1.6 %)	1 (1.6 %)	-	1 (1.6 %)
C. albicans	1 (1.6 %)	-	1 (1.6 %)	-	-	-
Other yeast species *(Cryptococcus curvatus, Trichosporon ovoides, Rhodotorula mucilaginosa Geotrichum candidum)*	21 (33.8 %)	3 (4.8 %)	24 (38.7 %)	22 (35.4 %)	3 (4.8 %)	25 (40.3 %)
Total	53 (85.4 %)	9 (14.5 %)	62	53 (85.4 %)	9 (14.5 %)	62

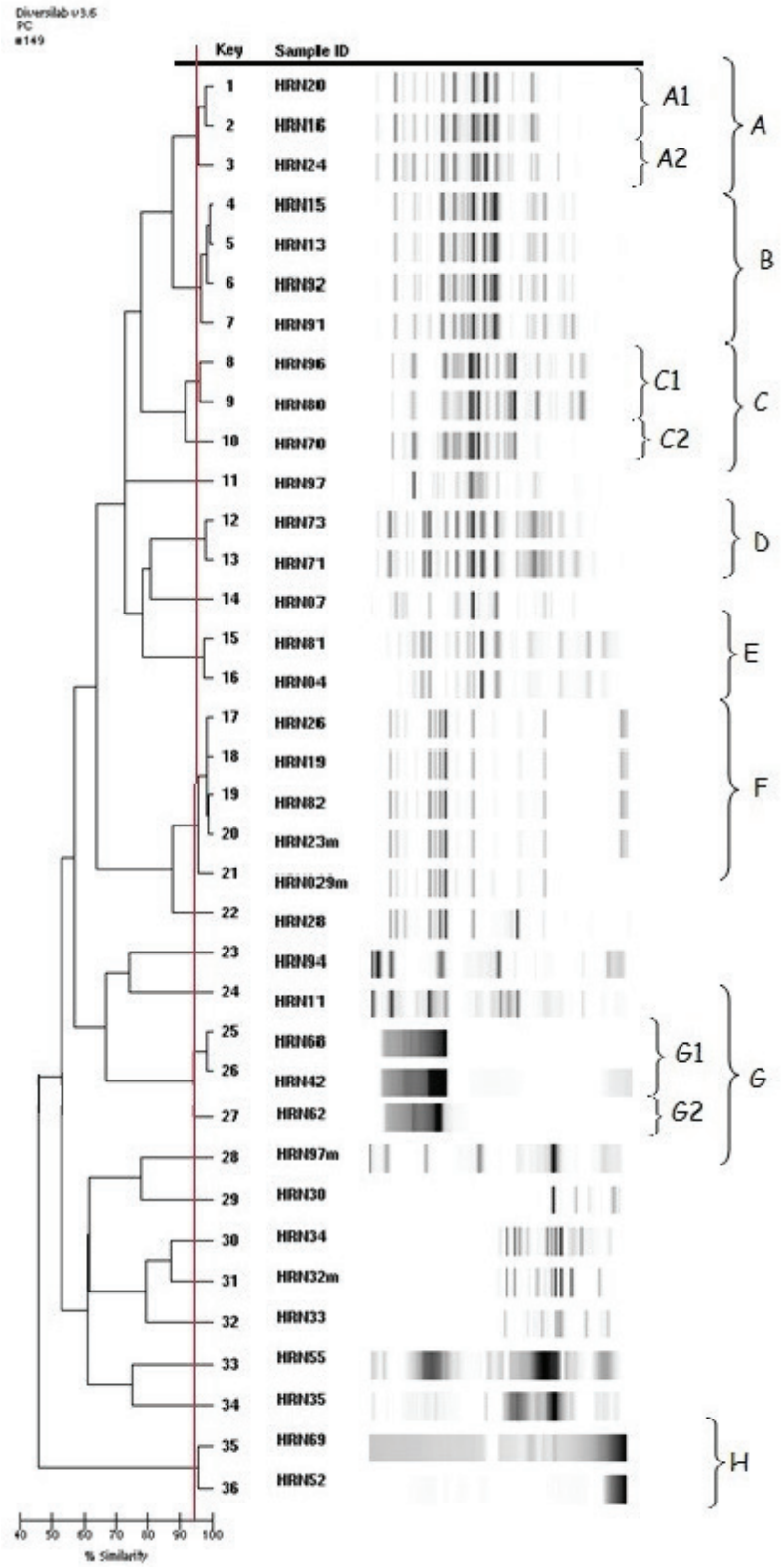

FIGURE 2. Dendrogram of Rep-PCR profiles for 36 *Candida* spp. isolates

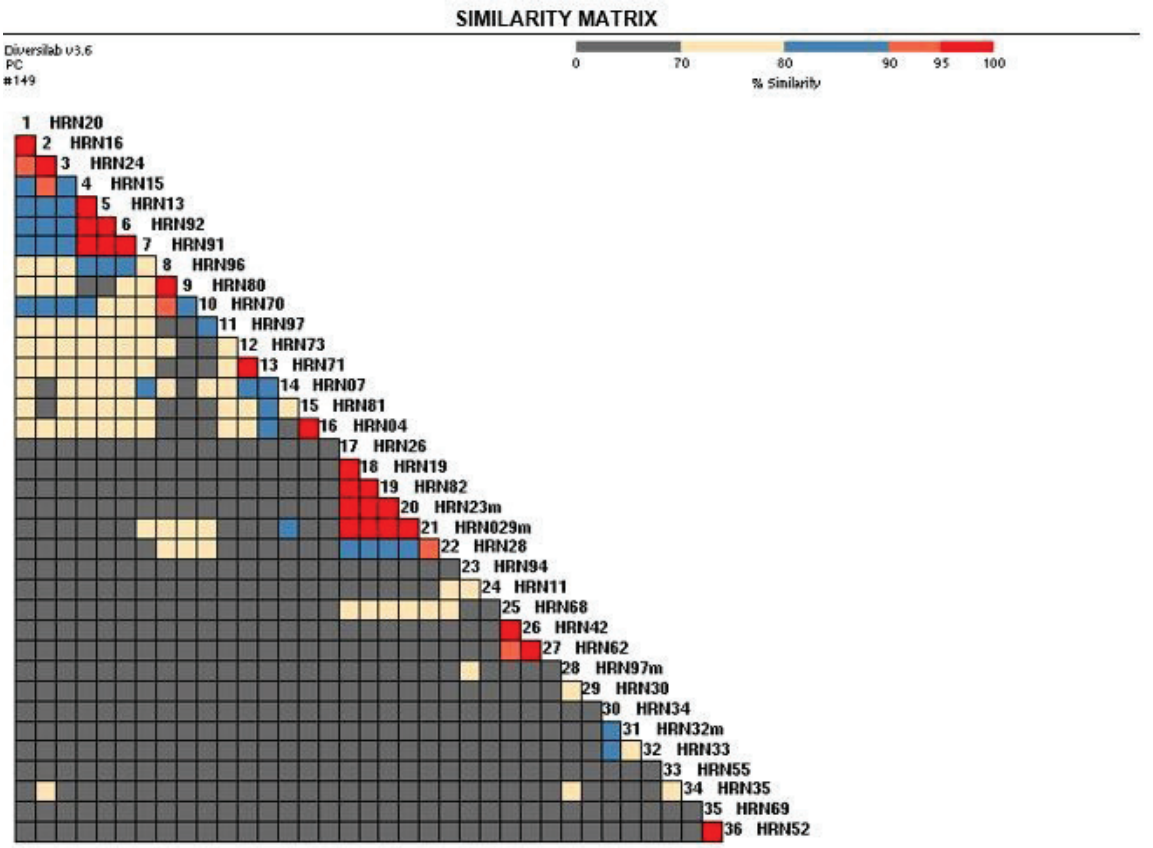

FIGURE 3. The similarity matrix of 36 *Candida* spp. isolates

Conclusion

A total of 37 (with one being lost) milk isolates obtained from healty and mastitic cows in Kayseri were identified as containing *Candida* species according to phenotypic characteristics as tested by, MALDI-TOF MS and VITEK-2. The most frequent *Candida* species found in this study was *C. lusitaniae* followed by very low frequency of other NAC species. No *C. albicans* was isolated in this study. Identification of NAC isolates from mastitic cows in this study might be associated with their possible pathogenic role in mycotic mastitis in dairy farms. Therefore, NAC species must be considered in the etiology of mycotic mastitis.

Strong antifungal resistance of *C. lusitaniae* isolates point out the need of in vitro susceptibility studies to evaluate the efficacy of antifungals, before starting the treatment of mycotic mastitis.

NAC isolates were also determined in healthy cow milk in this study. In order to produce healthy milk and milk products, required hygienic measures should be taken at all manufacturing stages from raw material to final product to prevent contamination.

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Klonska raznolikost i antifungalna osjetljivost vrste Candida spp. izolirane iz mlijeka

Sažetak

Cilj ovog istraživanja bila je izolacija, identifikacija, filogenetska analiza i analiza antifungalne podložnosti vrste *Candida* spp. iz uzoraka mlijeka zdravih i mastitičnih krava muzara s područja Kayseri u Turskoj. Uzorci mlijeka izuzeti od 300 krava analizirani su pomoću California Mastitis Test-a na prisutnost mastitisa. *Candida* spp. izolirana je pomoću selektivne podloge Brillance *Candida* Agar Base. Za potvrđivanje dobivenih izolata korišteni su fenotipski testovi, MALDI-TOF masena spektrometrija i VITEK-2 analize. Rezistencija izolata na fungicide fluktozin, flukonazol i kaspofungin testirana je primjenom Etesta i VITEK-2. Genetski homolozi izolata vrste *Candida* spp. određeni su primjenom RepPCR metode. U ovom istraživanju ispitivana su 62 izolata (53 od zdravih, 9 od mastitičnih krava) kvasca, a 37 (59,6 %) ih je identificirano da ne pripadaju vrsti *Candida albicans* (NAC). Fenotipski testovi pokazali su da od 62 izolata, njih 29 (46,7 %), 4 (6,5 %), 3 (4,8 %), 1 (1,6 %) i 24 (38,7 %) identificirani su kao sojevi *Candida lusitaniae, Candida catenulate, Candida tropicalis, Candida silvicola* te neke druge vrste kvasaca. Samo je jedan uzorak (1,6 %) identificiran kao *Candida albicans* pomoću metode MALDI-TOF, no nije potvrđen i metodom VITEK-2. Prema testiranju antifungalne osjetljivosti metodom VITEK-2, jedan (2,7 %) od izolata je bio rezistentan na flukonazol, jedan (2,7 %) na kaspofungin, i 4 (10,8 %) bili su rezistentni na flucitozin. Međutim, primjenom E testa, 10 izolata (27 %) bilo je rezistentno na flucitozin. Primjenom Rep-PCR metode utvrđeno je osam genotipskih klonova. Najčešći klonovi određeni u ovom istraživanju bili su genotipi F (13,8 %) i G (13,8 %; 2 podtipa). Zaključno, prisutnost NAC vrsta utvrđena je u uzorcima mlijeka i zdravih i mastitičnih krava. Potrebno je provesti epidemiološke studije kako bi se efikasno utvrdio glavni izvor istih te kako bi se razumjela raznolikost i distribucija klonova. Nužno je uzeti u obzir potencijalne rizike kontaminacije mlijeka kvascima s aspekta zaštite javnog zdravlja. Stoga je prijeko potrebno fokusirati se na odgovarajuće procedure sanitacije i osiguravanje adekvatnih uvjeta skladištenja mlijeka.

Ključne riječi: **antifungalna rezistencija, MALDI-TOF, VITEK-2, molekularna tipizacija, mlijeko, NAC (ne albicans) sojevi *Candida* vrste**

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