Molecular Diagnosis of *Candida* Species Isolated from Cases of Subclinical Bovine Mastitis

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ABSTRACT

Cattle mastitis is one of the most common bovine diseases and has an important role in the milk industry. It's considered that among all pathogens responsible for bovine mastitis, yeasts which belong to *Candida* genus, should be taken into account as an etiological agent. The aim of this study was to isolate *Candida* species from dairy cows which were suspected of subclinical mastitis and to identify these isolates by using conventional methods, chromogenic agar and sequence analysis. The material of the study consisted of 400 milk samples from 280 subclinical mastitic cows collected from 20 dairy cattle farms in Aydin region in Turkey. It was found that 96 of 400 (24.0%) samples were considered as positive for yeast isolation. Out of 96 isolates, 16 (16.7%) were identified as *Candida albicans* while 80 (83.3%) isolates showed non-albicans by the conventional method. By using chromogenic agar, identification was not achieved for 49 (51.0%) isolates. On the other hand, out of 96 isolates, 21 (21.9%), 17 (17.7%), 9 (9.4%) were identified as *Candida krusei, C. albicans, Candida tropicalis*, respectively. Nine species of yeast were isolated including *Candida parapsilosis* 22.9% (22/96), *Candida krusei* 21.9% (21/96), *Candida keyfr* 19.8% (19/96), *C. albicans* 16.7% (16/96), *C. tropicalis* 9.4% (9/96), *Candida glabrata* 4.2% (4/96), *Candida guilliermondii* 3.1% (3/96), *C. lipolytica* 1.0% (1/96), *Trichosporon asahii* 1.0% (1/96) by sequence analysis. Results showed that conventional method was appropriate for *C. albicans* while chromogenic agar displayed advantages in the isolation of *C. krusei, C. tropicalis,* and *C. albicans*. It was determined that molecular methods were suitable for all *Candida* species. In this study, it was demonstrated that *Candida* species, which were unfamiliar, could play a role in the etiology of bovine subclinical mastitis while leading yeast strains causing the infection in this region were non-albicans *Candida* species.

Key words: *Candida*; Subclinical Mastitis; Identification; Chromogenic Agar; Conventional Methods; Sequence Analysis.

INTRODUCTION

Candida fungi, known to be zoonoses, are opportunistic pathogenic microorganisms found in the normal flora of humans and animals (1). Approximately 200 *Candida* species are present, however there are a limited number of species that are pathogenic in living organisms (2).

Candida species are often isolated from mycotic bovine mastitis, but there is a significant increase in subclinical infections caused by non-albicans species including *Candida zeylanoides, Candida norvegica, Candida viswanathii, Candida guilliermondii*, and *Candida tropicalis* (3). In the same study, it was reported that *Candida albicans* was isolated in only 3.9% of clinical and subclinical mastitic cows.

The use of antibiotics and steroids for a long period of time for the treatment of mastitis increases the incidence of mycotic mastitis in dairy farms. In order to determine the treatment method for mastitis cases, a quick and reliable identification method is essential. Identification of *Candida*

species by conventional methods is accepted as gold standard method, which is cheap and reliable. However, conventional method has some important disadvantages. Some species cannot be fully identifiable and the identification process takes a longer time.

Most of *Candida* species on Sabouraud Dextrose Agar (SDA) are creamy and oval. Colony color is indistinguishable by using these media (1,2). For this reason, several chromogenic agars have been developed in recent years to provide rapid yeast identification. In this genus-specific chromogenic substrates containing medium, these substrates react with enzymes produced by yeast to provide the formation of colonies in various colors. The use of chromogenic medium facilitates recognition of specimens containing mixtures of yeast species and isalso suitable for primary isolation of yeasts (4).

Polymerase chain reaction (PCR) based methods for the rapid detection and identification of *Candida* species have also been described such as restriction fragment length polymorphism analysis (5), PCR-restriction fragment length polymorphism analysis (6) and random amplification of polymorphic DNA analysis (7). However, the use of these methods in clinical laboratories is impractical (8). The internal transcribed spacer (ITS) region in the ribosomal RNA (rRNA) for fungi is considered as formal fungal barcode (9). The rRNAs on genomic DNA form consecutive sequenced repeats of conserved gene regions. In particular, the ITS1 sequences between 18S and 5.8S and the ITS2 sequences between 5.8S and 28S shows more nucleotide base change than other rRNA gene regions. In addition, these molecular markers are safe because they can be standardized among laboratories, and are not affected by environmental conditions (8, 9, 10). It has been reported that differences in the sizes of the ITS2 regions of fungi are useful for the rapid identification of clinically important fungi (10, 11).

The aim of this study was to determine the prevalence of *Candida* species in subclinical mastitis and the identification of the isolates comparatively by different methods (conventional, chromogenic agar and sequence analysis).

MATERIAL AND METHODS

Clinical Examination

In this study, California Mastitis Test (CMT) was used by veterinarians to detect subclinical mastitis. The procedures and interpretations have been described previously (12).

Milk Samples

Samples were taken from cows which had not undergone any treatment for least one month. A total of 875 dairy cows were examined for suspicious subclinical mastitis in 20 different farms in Aydin region in Turkey. Out of 875 cows, 280 cows were diagnosed as subclinical mastitis and a total of 400 milk samples were taken from these mastitic cows. Milk sample distribution were as follows: 1, 2, and 3 mastitic milk samples were collected from 180, 80, and 20 cows, respectively. All dairy farms ranging in size from 5 to 50 animals were scaled as small to medium-sized. They all used milking machines and the age of the cows varied between 2 to 9 years.

Sample Collection

A total of 400 milk samples from CMT positive 280 cows were taken under aseptic conditions. During the collection, the following procedure was followed: teat ends were cleaned using 70% alcohol moistened swabs and allowed to dry. After discarding the first few streams, 2-5 ml of the milk samples were collected into 5 ml sterile glass flasks. Samples were refrigerated at 4°C during transportation to the laboratory and examined as soon as possible.

Mycological Examination

Milk samples were centrifuged at 3500 rpm for 5 min and the supernatant was discarded. The sediment was vortexed and a loopful streaked on Sabouraud Dextrose Agar (Merck, Germany). After incubation at 37°C for 72 hours, yeast samples were evaluated by Gram staining and examined microscopically for determining their stain and morphological characters which appeared as distinctly round, oval or elliptical. Yeast identification was performed according to Barnett *et al.* (13) and were stored in Sabouraud Broth (SB) (Becton Dickinson) containing glycerol 20% at -20°C.

Germ Tube Test

The Germ Tube Test is the first step in the identification of candidates. It is a simple and very valuable test that gives fast results, easy to apply and allows *C. albicans* to be separated from other candidates. For the test, a small amount of the yeast colonies was suspended in a test tube containing 0.5 ml of rabbit serum. The test tubes were incubated for 3 hours at 37°C. A drop of yeast-serum suspension was placed on a slide. Germ tube formation of *C. albicans* is considered to contribute to pathogenicity. Germ tubes were seen as

hyphae-like extensions when yeast cells transformed into their mature form (14).

Streaking to Chromatic (Liofilchem®, Italy) *Candida* Agar

The isolates and the standard strains (*C. albicans* ATCC 90028, *C. krusei* ATCC 6258, *C. tropicalis* ATCC 750) were streaked to SDA one day before. After 24 hours of incubation, growing strains was streaked on to chromatic *Candida* agar. Strains were incubated at 37°*C* for 72 hours for identification. After 24, 48 and 72 hours of incubation, colony color and morphology were evaluated by three different people. The preliminary evaluation was performed as recommended by the manufacturer (*C. albicans* green, *C. tropicalis* blue, *C. krusei* pink) (15).

DNA Extraction

DNA extraction from *Candida* spp. was performed by using a commercial genomic DNA extraction kit Fermentas (Thermo Fisher Scientific, UK) as recommended by the manufacturer company after DNA purity check and quantification. The ratio of OD260/OD280 (1.8-2.0) showed that DNA was pure (16). Two µl of supernatant was used as the DNA template in the PCR.

PCR

For species-level identification of *Candida* isolates, universal primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTG ATATGC-3') targeting the ITS2 region were used (11). Each PCR test was performed in a reaction mixture of 50 µl final volume containing the following components: 5 µl Taq buffer with Mg+ (10X), 1 µl of dNTPs (10 mM), 0.2 µl of each primer (100 pmol), 0.3 µl Taq DNA polymerase (5 U/µl) (ABM, Canada) and 2 µl of each template DNA and with the remaining volume as water. The DNA was amplified using the following protocol: an initial denaturation at 94˚C for 3 min, followed by 35 cycles of denaturation (94˚C for 30 s), annealing (55˚C for 30 s) and extension (72˚C for 1 min), with a single final extension of 7 min at 72˚C. The PCR reactions were run on a 2% agarose gel for 45 min at 100 V and stained with Safe View (ABM, Canada). After electrophoresis, the gel was placed in the chamber of the transilluminator device connected to the computer, and photographed under UV light. After PCR products were visualized by electrophoresis, the assessment was made as previously reported (11). Positive (*C. albicans* ATCC 90028, *C. krusei* ATCC 6258, *C. tropicalis* ATCC 750) and negative (*E. coli* ATCC 25922) strains were used as controls.

Sequence Analysis

In order to identify species at the level of our isolates, the amplicons were sent to a specialized company (Macrogen, South Korea) for sequence analysis. Sequence analysis was performed following purification procedure. Then, these sequences were compared with the gene bank in order to determine the *Candida* species. The nucleotide-nucleotide BLAST program was used for this purpose. The highest homology type (>97%) was considered as the type of sequence detected.

Sensitivity and Specificity

Sensitivity was expressed as the number of true-positives 100/the number of true-positives plus the number of false-negatives, and specificity was expressed as the number of true-negatives 100/number of true-negatives plus the number of false-positives (17).

RESULTS

Isolation

In this study, 96 (24.0%) yeast isolations were performed out of 400 milk samples.

Identification

Sequence analysis, conventional method (Gram stain and Germ tube formation), and chromogenic agar method were used for the identification of *Candida* species.

Sequence Analysis: The PCR products amplified from yeasts appeared as single bands from 200 to 500 bp on a 1% agarose gel (Figure 1).

These sequences were compared with the gene bank in order to determine the species. According to the sequencing results, nine species of yeast were isolated including *C. parapsilosis* 22.9% (22/96), *C. krusei* 21.9% (21/96), *C. keyfr* 19.8% (19/96), *C. albicans* 16.7% (16/96), *C. tropicalis* 9.4% (9/96), *C. glabrata* 4.2% (4/96), *C. guilliermondii* 3.1% (3/96), *C. lipolytica* 1.0% (1/96), *Trichosporon asahii* 1.0% (1/96) (Figure 2).

Conventional Method: As a result of Gram staining.

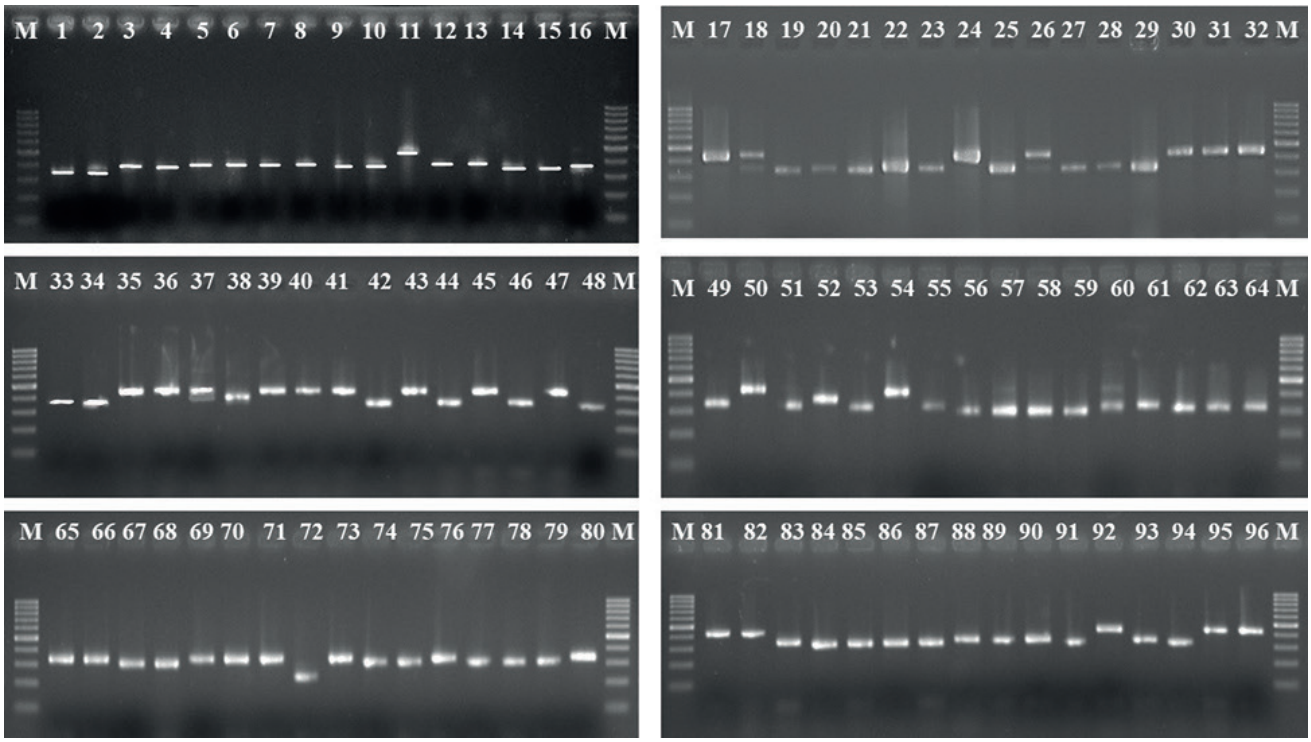

Figure 1: Gel electrophoresis images of isolates. M: 100 bp DNA Lader 1-96: Field isolates

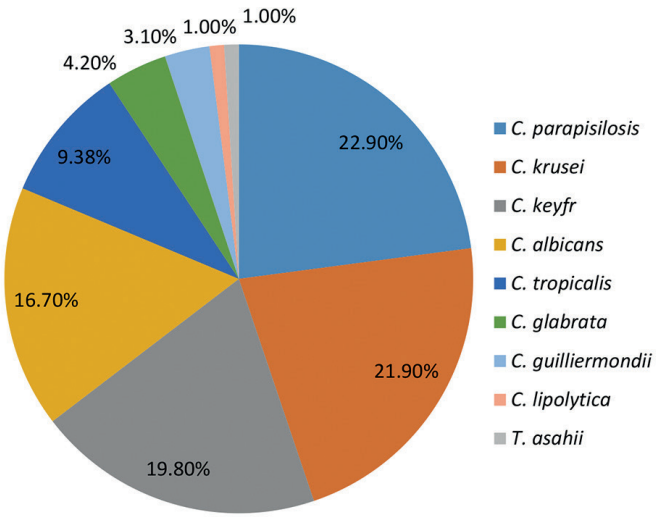

Figure 2: Distribution of *Candida* isolates according to sequence result

usually oval shaped yeast cells, which were Gram positive, were defined as *Candida* species. Germ Tube Test revealed that 16.7% (16/96) of the *Candida* isolates were germ tube positive and these isolates were identified as *C. albicans*. The remaining 80 (83.3%=80/96) non-germ tube formation isolates were identified as non-*albicans* species.

Chromatic Candida Agar: The definitive colony appearance was obtained for all species only after incubation for 48 h, and no differences in colony appearance was detected when the incubation was extended to 72 h. Isolates passaged on to chromogenic *Candida* agar were evaluated macroscopically according to colony morphology and colour. According to this examination, 21.9% (21/96) of the isolates in the chromogenic agar formed pink coloured R type colonies (*C. krusei*) and 17.7% (17/96) of pale green coloured S type colonies (*C. albicans*), 9.4% (9/96) of green-blue S-type colonies (*C. tropicalis*). From these findings, 49.0% (47/96) of the isolates could have been identified using the chromogenic *Candida* agar while 51.0% (49/96) of the isolates could not.

Comparison of Results

Using the conventional method, 16.7% (16/96) of the isolates were identified as *C. albicans* while 83.3% (80/96) were non-albicans species. Out of 96 isolates, 47(49.0%) were identified by using chromogenic *Candida* agar while 49 (51.0%) could not. All isolates were identified by sequence analysis successfully.

After the verification with sequence analysis, it was understood that all of the 16 *C. albicans* isolates were correctly identified by the conventional method. With respect to the sequence results of the conventional method for positivity of *C. albicans,* sensitivity and specificity were calculated as 100% and 100%, respectively.

It was determined that the chromogenic agar correctly identified 46 of the 47 isolates according to sequencing analysis. With respect to the sequence results of the chromogenic agar method for positivity of *C. albicans,* sensitivity and specificity were calculated as 100% and 98.7%, respectively. One growing colony with green colour on chromogenic *Candida* agar was evaluated as *C. albicans*. However, as a result of the sequence analysis, it was determined that this isolate was *T. asahii*. Nevertheless, both sensitivity and specificity for *C. krusei* and *C. tropicalis* were calculated as 100%, 100% respectively. Apart from these three species (*C. albicans*, *C. tropicalis* and *C. krusei*) on the chromogenic *Candida* agar, other *Candida* species were not distinguishable at the species level according to their growth (Table 1).

Table 1: Comparison of identification results with conventional method, chromogenic agar and sequence analysis

Isolation Result	Gram Stain/Germ Tube (Number of positive isolates)	Chromogenic Agar (Colony Color)	Sequence Analysis
C. parapsilosis	+/-	Not identified (Dirty white-Beige)	22
C. krusei	+/-	21 (Pink)	21
C. kefyr	+/-	Not identified (Dirty white-Beige)	19
C. albicans	+/+ (16)	17 (Light green)	16
C. tropicalis	+/-	9 (Green-Blue)	9
C. glabrata	+/-	Not identified (Dirty white-Beige)	4
C. guilliermondii	+/-	Not identified (Dirty white-Beige)	3
C. lipolytica	+/-	Not identified (Dirty white-Beige)	1
T. asahii	+/-	* (Green)	1

* An isolate has been identified as *C. albicans* according to the chromogenic agar because it shows a green color on a chromogenic agar. However, the isolate, which is compatible with the sequence ending *Trichosporon asahii*, and the negative result of the germ tube test support the sequence result.

DISCUSSION

Candida species are fungi frequently isolated from the mastitic cows' milks, especially those who have been treated with intensive antibiotics or whose immune system have been suppressed for various reasons. Identification of *Candida* species has great importance due to the differences in the antifungal therapeutic response among the species within the genus *Candida*.

It was reported that the prevalence of udder infections originated from *Candida* spp. in recent years was lower compared to other microorganisms (18). It was also reported that there was a significant increase in important *Candida*-related mastitis cases in various countries (19, 20, 21). For example, China (19) and Algeria (20) revealed the frequency of *Candida* which was 79.4% and 71.9%, respectively. In the present study, the rate of *Candida* isolation was found as 24.0%. Similar results were obtained by Zaragosa *et al.* (3) in Mexico (25.7%), Eldesouky *et al.* in Egypt (27.3%) (22), and Costa *et al.* (23) in Brazil (29.35%).

The germ Tube Test has been used for many years to provide rapid identification of *C. albicans* from clinical isolates. It is a simple and highly sensitive test which is also used presently. By using this test, *Candida* species isolated from the clinical material can be distinguished as either *albicans* or non-*albicans* in the first stage. Incidentally, the germ tube test can give false negative results in 5% of samples with antifungal therapy and immunodeficiency. Apart from *C. albicans*, *C. stellatoidea* and *C. dubliniensis* are germ tube positive (2, 14). In this study, germ tube test was applied to isolated *Candida* species and it was determined that 16 *C. albicans* were all positive according to the results of the germ tube test. For the positivity of *C. albicans* in *Candida* isolates, sensitivity and specificity were calculated as 100% and 100%, respectively according to the sequence results of the germ tube test. These findings were found to correspond with the findings of other researchers who had reported that the specificity and sensitivity of the germ tube test was high (24).

Another method used in the identification was the chromogenic agar method which is particularly preferred in terms of its easy application, rapid yielding and species-level

identification. The chromogenic *Candida* agar used in this study was specifically designed to distinguish *C. albicans*, *C. tropicalis* and *C. krusei* species from others. However, due to the identification of one of the colonies as *T. asahii* instead of *C. albicans* from 17 colonies grown on the chromogenic agar with green color, reduced the success of the chromogenic agar. This misidentification may have been arisen from the fact that *Trichosporon* spp. is very similar to the colony colours of *C. albicans* (25). Sensitivity and specificity of *C. albicans* were calculated as 100% and 98.7%, respectively, according to the sequence results of the chromogenic agar method. Both sensitivity and specificity were calculated as 100% for *C. krusei* and *C. tropicalis* according to the sequence results of the chromogenic agar method.

The distribution of *Candida* species shows diversity in several countries. Krukowski *et al.* (26) reported that the most frequently isolated species in Poland were *C. kefyr*, *C. cirferi*, and *C. krusei*. In another report, the most frequent speices were *C. krusei*, *C. rugosa*, *C. kefyr*, *C. albicans*, and *C. tropicalis*. In the same study, it was reported that less common isolates were: *C. zeylanoides, C. parapsilosis, C. guilliermondii* (27). Similarly, another study in Poland (28) reported that *C. parapsilosis* was the most dominant *Candida* species they isolated from bovine mastitis, followed by *C. krusei*.

In our investigation, the most frequently isolated species were *C. parapsilosis* followed by *C. krusei*, *C. keyfr*, *C. albicans*, *C. tropicalis*, *C. glabrata*. *C. parapsilosis*, although initially considered nonpathogenic, is more widely studied than *C. albicans. C. parapsilosis* has an important impact on public health, since it leads to invasive fungal infections causing serious morbidity and mortality (29). The present study showed a clear overall predominance of non-*albicans* among the *Candida* species. This finding confirmed the tendency observed in another study conducted in China (19), in contrast to the other report which described high prevalence of *C. albicans* in mastitic cattle (22).

In this study, *T. asahii* isolated from mastitic cattle milk was at a very low rate 1.0%. *Trichosporon* species, another yeast, have been reported as a cause of fungemia, especially in patients who have neutropenia and cancer (30). This study was parallel to the others which reported that *T. asahii* was isolated at a low rate in mastitic cattle milk (19, 31).

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 (32). In particular, PCR has increasingly been used for *Candida* diagnosis, as it is quick, simple, specific, sensitive and reliable (33). In the last two decades, the DNA sequencing method has revolutionized the characterization of microbial relationships. ITS regions have proven to be reliable target regions for amplification and sequencing of the distinction of important yeast species. Protected ITS regions that do not encode can be used to distinguish fungal pathogens because of their rapid evolution. Discrepancies between phenotypic and genotypic methods can be solved with sequence results of the ITS 2 region. The ITS 2 sequence polymorphism has been reported to be very useful in the identification of pathogen species and in the classification of new pathogen species (8, 11). In the present study, nine different yeast speices (*C. parapsilosis*, *C. krusei*, *C. keyfr*, *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. guilliermondii*, *C. lipolytica, T. asahii*) were identified with using primers targeting the ITS 2 region.

Consequently, despite the fact that conventional methods used in routine laboratories are inexpensive, reliable and still considered as gold standard, identification with those is not sufficient to distinguish *Candida* species. However, they may be used for the rapid identification of *C. albicans* and some non-albicans species (*C. krusei*, *C. tropicalis*), which are clinically important. Finally, it can be said that in cases where rapid and reliable identification is required, sequence analysis is more beneficial.

ACKNOWLEDGEMENTS

This manuscript was derived from the first author's PhD Thesis, supported by Aydin Adnan Menderes University Scientific Research Projects Unit (Project Number: VTF-15001) and the authors would like to thank Prof. Dr. Bulent Bozdogan (Aydin Adnan Menderes University, Medical Faculty, Department of Medical Microbiology, Aydin, Turkey) for his help and support.

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