



Conventional and Molecular Identification of Mycotic Mastitis Caused by *Candida* in Farm Animals

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ABSTRACT

Mycotic mastitis due to *Candida* is increasing and the identification of *Candida* species is crucial in choosing the ideal antifungal drugs. Identification of *Candida* to species level is essential as many non-albicans *Candida* developed drug resistance against antifungal agents. A total of 138 milk samples (24, 15, 46 and 53 from goats, sheep, buffaloes and cows, respectively) were streaked on plain plates of SDA and incubated. CHROM agar was used to differentiate *Candida* isolates. Mycological examination revealed the presence of yeast in 8.3, 6.7, 32.6 and 47.2% milk samples of goats, sheep, buffaloes and cows, respectively. The total isolation percent of yeast in all milk samples was 31.2%. Species identification of *Candida* in the present work using both CHROM agar and the bio-typing assay (API 20 C AUX) revealed the highest presence of *Candida albicans* (11.6%) followed *C. glabrata* (5.8%), *C. tropicalis* (5.1%) and *C. krusei* (2.9%). Multiplex PCR gave two different sized PCR products specific for every tested *Candida* isolates separately. It was concluded that phenotypic and biochemical assays are time-consuming and exposed to misdiagnosis. Whereas multiplex PCR is a genotypic-based technique that permits the identification of species that is difficult to be identified conventionally because of their abnormal morphology or biochemical features and also this assay is simple, easy and time saving so it is recommended to be implemented in clinical laboratories.

Key words: *Candida*, Milk, API 20 C AUX, CHROM agar, PCR.

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INTRODUCTION

Raw milk provides a good environment plus all basic nutrients for the growth of various fungal species (Callon et al. 2007). Those microbes can contaminate raw milk during different processing procedures and in addition to this, it is affected by the animal's physiological state, weather and breeding circumstances (Gulbe and Valdovska 2014).

Bovine mastitis is a disease that involve several causes including the relationship between the host, pathogen and the environment. It is one the most common and infectious manifestation of dairy cows. It is also of economic value in the whole world as it causes \$35 trillion losses in the global dairy industry (Bradley 2002).

Mastitis is one of the first three pathologies of dairy animals. This pathology may not have clinical manifestation (latent infection, subclinical mastitis), it is the most frequent (95 to 98% of the cases) and can have an obvious sign (clinical mastitis), the latter only represents 2 to 5% of the cases. Studies on fungal mastitis are becoming more abundant due to their increasing prevalence. (Akdouche et al. 2018). In mastitis, mammary gland

undergoes immune response in which the permeability of conjunctions between milk and blood (milk-blood barrier) are disrupted and this cause physical change in milk (Youssif et al. 2020; Gao et al. 2020; Mingala et al. 2020).

Researchers have revealed that fungal mastitis is increasing, and the most commonly derived pathogens are *Candida* species which are found normally in the surroundings of dairy cattle (Mbuk et al. 2016). Inadequate antibiotic treatment course can result in outbreaks of fungal mastitis because of the increase of latent infection during the drying-off stage of animals (AL-abidy et al. 2019). *Candida* species are the most known cause of mycotic infections globally. They are considered the third most common etiology of healthcare-associated infections. Although *Candida albicans* is the most common species, non-albicans *Candida* begin to increase remarkably during the last twenty years (Taei et al. 2019).

Identification of *Candida* species is of great importance in choosing the ideal antifungal drug and as a consequence the treatment process will occur properly as the response against antifungal drugs vary between different *Candida* species. If complete identification is not

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done, random antifungal will be used. The random and abundant use of antifungal drugs for prophylaxis and treatment can develop resistance against some *Candida* species. Identification of *Candida* to species level is crucial as many non albicans *Candida* have lowered susceptibility against antifungal agents (Erbaş et al. 2017).

CHROM agar is a nutritive agar medium with chromogenic substances that can generate different color for each *Candida* species as light to medium green color for *C. albicans*; dark blue to metallic blue color for *C. tropicalis* finally pink color surrounded by whitish edge for *C. krusei*. The rest of yeast isolates might generate light to dark mauve or cream colors (e.g., *C. glabrata*). CHROM agar medium can successfully differentiate *C. albicans*, *C. tropicalis*, and *C. krusei* (Ozcan et al. 2010). API 20 C AUX is a strip that can be used to perform 20 different biochemical tests sufficient for the identification of a single *Candida* isolate in addition to this it included a control negative and a control positive. Correct results were achieved when the profile gives the accepted match with the manufacturer's criteria. (Gündeş et al. 2001).

Phenotypic and biochemical assays are widely used in developing countries for species identification (Posteraro et al. 2015). On the other hand, these methods need long time for preparation and are exposed to mistakes (Kathuria et al. 2015). Recently, variety of molecular assays have been utilized for *Candida* species identification and they regarded accurate and easy to utilize (Liguori et al. 2010). In the last decade, polymerase chain reaction (PCR) is considered as a standard platform in several clinical laboratories even in developing countries due to its reproducibility and affordability. (Arastehfar et al. 2019). Multiplex-PCR was used for amplification of the DNA of *Candida*. It was used to amplify more than one sample in a single reaction (Hermansyah et al. 2017). Therefore, our goal in the present work was performing quick differentiation of *Candida* species isolated from milk samples to species level using a quick and easy PCR-based technique that can identify four *Candida* species (*C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei*). This assay combined two yeast-specific primers and four *Candida* species-specific primers in a single PCR process giving two different sized bands specific for each species.

MATERIALS AND METHODS

Yeast Isolates Isolation and Identification

A total of 138 milk samples (24, 15, 46 and 53 from goats, sheep, buffaloes and cows, respectively) were streaked on plain plates of SDA and were incubated at 30°C for 72h. Pure colonies were subjected to morphological and differential biochemical identification assays (Marinho et al. 2010). Germ-tube test in which yeast isolates were inoculated into 0.5ml of serum and were incubated at 37°C for 2h. After this period, a drop was examined microscopically for a tube projected with straight walls and without constriction. Germ-tube was positive for *C. albicans* and *C. dubliniensis*, Chlamydoconidia production test on rice agar tween 80 media on which the yeast isolates were streaked on the surface of the medium and a cover slide was added above the streaking lines after three days of incubation at 30°C. The plates were microscopically examined for the presence and for the arrangement of

chlamydoconidia and carbohydrate assimilation test for maltose, trehalose, xylose, galactose, lactose, sucrose and glucose since all the species of *Candida* can assimilate this carbohydrate. The plates were incubated at 37°C for 3-4 days. The existence of growth around the carbohydrate disc was considered as assimilation positive for this carbohydrate.

CHROM agar (Cat # 1382, Condalab, Madrid, Spain) was used to differentiate *Candida* isolates. It was prepared according to the manufacturer's instructions. Yeast isolates were inoculated by streaking and were incubated at 30°C and the judgment of the results was performed by two researchers independently, after 24, 48, and 72h of incubation. Fungal isolates were further identified using API 20 C AUX (Cat # 1006663720, Biomerieux, Marcy l'Etoile, France). It is a readymade 20 biochemical reactions included both control positive (glucose) and control negative in one disposable plastic strip sufficient for the identification of one *Candida* isolates and the assay was performed according to the manufacturer's instructions.

Molecular Identification of *Candida* Isolates

Genomic DNA Extraction was performed with (DNeasy Plant Mini Kit) according to the manufacturer's protocol.

PCR Amplification

Amplification reaction was done using both universal yeast primers (UNI1 and UNI2) and *Candida* species-specific primers previously described by Carvalho et al. (2007). Multiplex PCR amplification reaction was performed in a 30µl volume consisting of master mix 15µl, primers (UNI1 and UNI2, 0.55µM each; *C. albicans* and *C. krusei*, 0.15µM each; *C. glabrata* and *C. tropicalis*, 0.2µM each), 5µl DNA template and then complete the volume of nuclease free water. PCR was routinely carried out in thermal cycler (model 1105 TECHNE) under the following conditions: Initial DNA denaturation for 10 min at 94°C followed by 40 cycles at 94°C for 15s, 55°C for 30s, and 65°C for 45s and parallel to this control negative reaction was performed in which nuclease free water replaced the DNA template. Electrophoresis was performed for seven ml from each reaction tube with 2% agarose. Visualization of DNA bands was implemented by Ethidium bromide staining (0.5µg/ml) and imaging of the produced bands was implemented using a digital imaging system (White/UV transilluminator) and a 100-bp DNA ladder was used as a ruler to compare between the produced DNA products. Gel analysis was done by lab image software program version 2.7.0 Copyright © 1999-2003 Kapelan GmbH.

RESULTS

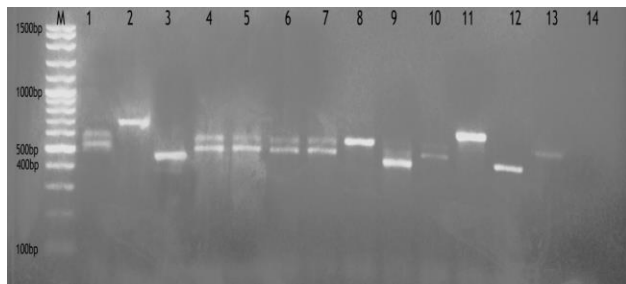
A total of 138 milk samples tested for mycological examination revealed the presence of yeast in 31.2% samples. Being positive for fungal growth in 8.3, 6.7, 32.6 and 47.2% milk samples of goats, sheep, buffaloes and cows, respectively (Table 1). Species identification of *candida* in the present work as shown in Table 2 that indicated the presence of *Candida albicans* in 11.6% while it was 2.9% positive for *C. krusei* whereas it was 5.8% for *C. glabrata* and finally it was 5.1% for *C. tropicalis*.

Table 1: Incidence of yeast isolates in the examined milk samples

Milk samples	Yeast positive samples	
	No.	%
Goat milk (n=24)	2	8.3
Sheep milk (n=15)	1	6.7
Buffalo milk (n=46)	15	32.6
Cow milk (n=53)	25	47.2
Total (n=138)	43	31.2

Table 2: Results of identification using CHROM agar

Species no.	Colony colors using CHROM agar			
	Green	Pink	White	Blue
Goat milk (n=24)	1	-	-	-
Sheep milk (n=15)	1	-	-	-
Buffalo milk (n=46)	5	2	2	3
Cow milk (n=53)	9	2	6	4
Total (n=138)	16 (11.6%)	4 (2.9%)	8 (5.8%)	7 (5.1%)

**Fig. 1:** Amplified PCR products (agarose gel electrophoresis) of different *Candida* isolates. (Lane M: 100-bp DNA ladder, Lanes from 1-13 are the tested isolates and Lane 14: negative control).

Accurate identifications were achieved for 35 *Candida* isolates with CHROM agar medium and API 20C Aux in the present work. *Candida* isolates identified conventionally were tested using multiplex PCR. Results of multiplex PCR in Fig. 1 showing the agarose gel electrophoresis pattern of thirteen *Candida* isolates. *C. tropicalis*, *C. glabrata*, *C. albicans* and *C. krusei* were identified in samples at molecular weight 500 and 580, 890, 440 and 570 base pair (bp), respectively.

DISCUSSION

Mastitis is one of the most dominant disease in dairy animals. The symptoms of mastitis include different abnormalities as the watery consistency of milk, clots, flakes, or pus in mastitic milk (Firyal et al. 2019; Saleem et al. 2019; Rawat et al. 2020). The incidence of mycotic mastitis is generally very low and the incidence of fungal mastitis in the United States ranged from (2%-7%) and those rates can be higher in tropical regions. But the prevalence of mycotic mastitis has remarkably increased during the past decade. Mycotic mastitis is often caused by *Candida* species (Sonmez and Erbas 2017).

The present study revealed the presence of yeast in 8.3% in goat milk samples, 6.7% in sheep milk samples, 32.6% in buffalo milk samples and finally 47.2% in cow milk samples while the total isolation percent of yeast in all milk samples was 31.2%. Those results found to be much similar to those obtained by dos Santos and Marin (2005) as fungi were isolated from 32% of the tested samples and *Candida* species represents 17.3% from them. Our results were higher than those obtained by Krukowski et al. (2001) as they assured that the isolation percent of fungi from the

tested milk specimen in the Lublin region in Poland was 9.6%. Moreover, Dworecka-Kaszak et al. (2012) isolated fungi from 14% of tested samples and they isolated *Candida* species with other microbes species from 39% but *Candida* species was isolated alone from 11% of these cases also similar results were achieved by Costa et al. (1993) in Brazil by 12.07%. Lower isolation rates were recovered by Awad et al. (1980) in Egypt with 6.1% while the lowest isolation rates with 1.3% in Denmark by Aalbek et al. (1994). However, Krukowski et al. (2020) isolated yeasts from (1%) of the tested specimens and this is very low isolation rate in compared with our study and the other studies as well and they derived *Candida* species from (91.6% of positive yeast specimens). dos Santos and Marin (2005) stated that in surveys, fungal isolation percentage implemented in many countries differs considerably (Imran et al. 2020).

Species identification of *Candida* in the present work as shown in Table 2 assured the presence of *Candida albicans* in (11.6%), *C. krusei* (2.9%), *C. glabrata* (5.8%) and *C. tropicalis* (5.1%). Those results found to be much similar to those obtained by Spanamberg et al. (2014) as they isolated fungi *C. albicans*, *C. glabrata*, *C. tropicalis* and *Pichia guilliermondii* from both normal and mastitic ewe milk samples. In the present investigation, *C. albicans* was the most frequently isolated species (11.6%), *C. glabrata* (5.8%), *C. tropicalis* (5.1%) and finally *C. krusei* (2.9%) and our results are in agreement with those obtained by Costa et al. (1993) as they isolated mostly *C. albicans* but different results were obtained by Dworecka-Kaszak et al. (2012) as they mostly isolated *C. parapsilosis* (25 strains), *C. krusei* (15 strains) and only one *C. albicans* strain and also Krukowski et al. (2001) assured that in Poland, *C. kefir*, *C. cirferi*, and *C. krusei* were the most abundantly isolated *Candida* species.

Accurate identifications with CHROM agar medium was achieved in the present work. However, those strains of *C. albicans* and non- *C. albicans*, sometimes did not only fail to produce the predicted colors, but sometimes the produced colors were also false. In particular, with CHROM agar medium *C. tropicalis* and *C. krusei* can be identified correctly, while the rest of the yeast species were difficult to be identified correctly. A laboratory technique that is performed within 72h for preliminary identification of *C. albicans*, *C. tropicalis*, and *C. krusei* is of doubtful value. Chromogenic medium could not be used as a sole identification tool as biochemical identification must be performed to avoid incorrect identification accordingly, CHROM agar medium could be considered as a primary isolation medium and cannot be used alone in identification of *Candida* species (Ozcan et al. 2010).

The API 20C AUX, a manual assay, needs great accuracy during test performing, experience in the judgment of turbidity level, and frequently, long incubation periods, but it is dependable, simple to use and cheap (Liguori et al. 2010).

Precise identification with API 20C Aux was accomplished in the present study. Several studies on the API 20C Aux system found that it has 88-99% accuracy. Our results were similar with those obtained by Fenn et al. (1994) as they obtained 99% accurate identification of their yeast isolates using API 20C Aux while Davey et al. (1995) achieved 88% correct identification and lower appropriate

identifications (87%) were obtained by Gündeş et al. (2001) and this variation may attribute to differences in species distribution. Incompletely identified isolates need additional tests to complete their identification.

Due to the lack of financial resources in developing countries for high-priced and accurate means of identification, such as MALDI-TOF MS and Sanger sequencing. Multiplex PCR assay is cheaper alternative with good outcome and can be implemented in large-scale epidemiological studies (Arastehfar et al. 2019).

Simple, easy and time saving method for identification of *Candida* species using both universal yeast primers (UNI1 and UNI2) and *Candida* species-specific primers. Yeast universal primers enable a broad detection ability for yeast isolates whereas species-specific primers could identify species of *Candida* in a sole PCR tube.

The combination of UNI1 and UNI2 with the species-specific primers of *Candida* implemented in this study yielded specific band patterns, enabling simple and rapid identification of the questionable *Candida* species. However, this technique is a genotypic-based method that can identify *Candida* species which is difficult to be differentiated by conventional methods and also it can identify mixed infections. Furthermore, this multiplex technique can be adopted in clinical laboratories as it is relatively cheap since it only requires the basic DNA isolation reagents and PCR components and it does not need neither restriction enzymes nor DNA probes.

Candida isolates were tested using multiplex PCR reaction in the current work and *C. tropicalis*, *C. glabrata*, *C. albicans* and *C. krusei* were identified successfully using the multiplex PCR and the produced PCR product bands shown in Fig. 1 were similar to the results obtained by (Carvalho et al. 2007). The multiplex *Candida* PCR had exhibited good sensitivity and specificity on clinical specimens of high-risk individuals for direct recognition of *Candida* species in whole blood specimens. Multiplex PCR technique can serve contribute rapid and precise diagnosis of candidiasis. (Fuchs et al. 2019) and also (Susilawati et al. 2019) assured that multiplex PCR assay is a quick alternative in discriminating *Candida* species due to its high specificity and high sensitivity.

Conclusion

Conventional ways used in *Candida* species identification are less effective and have a lot of drawbacks and Multiplex PCR performed in the present work enables us to identify accurately *Candida* species even those with abnormal characteristics and this technique should be executed in clinical laboratories.

Author's Contribution

All authors shared in study design, data gathering, executing the practical part, results analysis and shared in writing of the manuscript.

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