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Isolation, Biochemical Identification and Molecular Detection of Yeasts from Kareish Cheese

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Abstract: The present study was performed on a total number of 50 samples were collected from fresh kareish cheese. Yeasts were isolated with an incidence of 52 % from fresh kareish cheese samples. The isolated yeasts were identified as *Candida albicans* as well as *Geotrichum candidum* with an incidence of 19.25 %, *C. glabrata*, *C. guilliermondii*, *C. tropicalis* 7.7 % (for each species) *C. dubliniensis*, *C. krusei*, *C. parapsilosis*, *Cryptococcus neoformans*, *Trichosporon cutaneum* 3.85 % (for each species) and mixed yeast with an incidence of 19.15 %. Polymerase chain reaction (PCR) was used as a rapid and more sensitive method for detection of phospholipase as well as lipase enzymes genes as (virulent factors of *C. albicans* and *Geotrichum candidum* respectively).

Key words: Kareish Cheese · Yeasts-*Candida albicans* · G*eotrichum candidum* · Phospholipase · Lipase

INTRODUCTION

Karish cheese is one of the most popular local types of fresh soft cheese in Egypt. The increasing demand by Egyptian consumers is mainly attributed to its high protein content and low price [1]. Karish cheese is traditionally made from skim cow or buffalo's milk which is extracted directly into special earthenware pots known as (shalia) and kept undisturbed in a suitable place to allow the fat to rise to the surface forming a cream layer. Then the cream layer is removed and the curd is poured onto a mat which is tied and hung with its contents to allow the drainage of the whey. This process of squeezing takes two or three days until the desired texture of the cheese is obtained. Finally, the cheese is cut into suitable pieces and salted cheese is left for a few hours in the mat till whey no longer drains out, then it is ready to be consumed as fresh soft cheese [2]. This traditional method affords many opportunities for microbial contamination. It is generally made from raw milk often of poor bacteriological quality and produced under unsatisfactory conditions. Also, this product is sold uncovered without a container, thus the risk of contamination is very high. Therefore, it can be considered as a good medium for the growth of different types of spoilage and pathogenic microorganisms [3, 4]. It is widely recognized that yeasts can be an important component of the micro-flora of many cheese varieties because of the low pH, low moisture content, high salt concentration and refrigerated storage of these products [5]. Nevertheless, yeasts play a dual role depending on the cheese. In fact, in some cheese types they make a positive contribution to the development of flavor and texture during the stage of maturation, while in other varieties, yeasts can be regarded as spoilage organisms. Yeast spoilage is recognized as a problem primarily in fermented milk and cheese [6, 7].The sources of these yeast infections are located along the whole chain of production from the farm to the final product. There are numerous references concerning the significance of the presence of yeasts in dairy products, where they may contribute positively to the characteristic taste and flavor development during the stage of maturation or, on the contrary, may lead to product spoilage [8, 9]. In Egypt, the information about the involvement of Karish cheese in human illness and economic losses are unknown. On the last years, a considerable increase of illness caused by infection with *Candida* species, these yeasts has been related to animal variation species, due to the hazard use of antibiotics. There were several species of Candida such

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as *C. albicans, C. tropicalis, C. pseudotropicalis* and *C. krusi. C. albicans* is considered the most important pathogen responsible for infectious diseases in animals [10]. Therefore, this study was designed to enumerate yeast populations in raw and pasteurized Karish cheese milk samples through isolation and identification of the yeast species using both conventional method and advanced technique (polymerase chain reaction)

MATERIALS AND METHODS

Samples: A total of 50 collective kareish cheese milk samples were obtained from retail farmers and supermarkets in Giza Governorate. Each cheese sample was represented by one whole cheese (250 g). The samples were taken under possible aseptic condition in sterile bags and transferred in an ice box to the laboratory.

Isolation of yeast according to the method of Van der Walt and Yarrow [11]:

The rind and core samples (10 g) were placed in 99 ml of sterile 2% (w/v) sodium citrate preheated to 45°C and were homogenized using a Stomacher blender (Seward Stomacher for 90 s at 250 rpm [12]. Serial dilutions in 2% sodium citrate solution were applied and spread on Sabouraud's Dextrose agar plates (40.0 gm Dextrose; 10.0 gm peptone; 20.0 gm agar and 50.0 mg chloramphenicol per liter). The plates were incubated at 25°C for 3 days. Individual colonies were selected based on shape and color (smoothness of surface, regularity of border, consistency, color, etc.), streaked to single colonies on yeast potato dextrose agar media (1 % yeast extract, 2 % dextrose, 2 % peptone and 1.5 % agar), incubated for 5 days at 25°C and checked for purity. Counts for each individual type of colony were made in order to estimate the relative occurrence of the various yeasts present in the samples. Calculated Yeast species counts were done as number of colony forming units per gram of sample and were reported as log10 CFU g^{-1}, then kept at 4°C until the colonies were identified.

Complete identification of different yeast isolates was performed according to Cruickshank *et al*. [13], El-Eraqy [14], Makhlouf [15] and Khan *et al*. [16].

DNA extraction from C. albicans and *Geotrichum candidum isolates:* DNA was extracted from *C. albicans* and *Geotrichum candidum* isolates using extraction kit QIA amp mini kit (Qiagen), following the manufacturer instruction.

Polymerase Chain Reaction for detection of the PLB1-specific Region of Candida Albicans: Total genomic DNA isolated from *C. albicans* cells was subjected to PCR amplification with oligonucleotide primers targeting a 751 bp region representing the 59 half of the PLB1 gene, as described by Mukherjee [17]. The oligonucleotide primers (Biobasic inc.) used were,

Forward 5' ATGATTTTGCATCATTTG 3'
Reverse 5' AGTATCTGGAGCTCTAC 3'

PCR reaction mixture contained 5 µl of DNA template (20 ng/µl), 12.5 µl of 1× Master mix (25 mM $MgCl_2$, 10 mM dNTPs mix and 5 U/µl of *Taq* DNA polymerase), 1 µl of 10 pmol of each primer (Bioanalysis Centrosud, Italy),) and distilled water up to 25 µl final volume. PCR reactions were carried out in DNA Thermal cycler (Biometra, Germany) at an initial 5 min denaturation step at 94°C followed by 35 cycles of denaturation step at 94°C for 1 min, annealing step at 47°C for 1 min and extension step at 72°C for 1 min. After the 35 cycles, aliquots of 10 µl of amplified products were analyzed by electrophoresis on 0.9 % agarose containing 0.5 µg/ml ethidium bromide at 80 V for 90 min and PCR products were detected by UV transilluminator.

Polymerase Chain Reaction for detection of the lipase-specific region of
Geotrichum candidum:

Total genomic DNA isolated from *Geotrichum candidum* cells was subjected to PCR amplification with oligonucleotide primers targeting a 300 bp region as described by Sanio and Nilanjan [18]. The oligonucleotide primers (Biobasic inc.) used were,

Forward 5' GCCCTCTGCTAACAAGTCCTAC 3'
Reverse 5' TTTAAGTCGGGGCCCTTACCTA 3'

PCR reaction mixture contained 5 µl of DNA template (20 ng/µl), 12.5 µl of 1× Master mix (25 mM $MgCl_2$, 10 mM dNTPs mix and 5 U/µl of *Taq* DNA polymerase), 1 µl of 10 pmol of each primer (Bioanalysis Centrosud, Italy),) and distilled water up to 25 µl final volume. PCR reactions were carried out in DNA Thermal cycler (Biometra, Germany) at an initial 5 min denaturation step at 94°C followed by 30 cycles of denaturation step at 94°C for 1 min, annealing step at 56°C for 1 min and extension step at 72°C for 2 min then final extension step at 72°C for 10 minutes. After the 35 cycles, aliquots of 10 µl of amplified products were analyzed by electrophoresis on 0.9 % agarose containing 0.5 µg/ml ethidium bromide at 80 V for 90 min and PCR products were detected by UV transilluminator.

RESULTS

Percentages of Yeasts Isolated from Fresh Kareish Cheese: Fifty fresh kareish cheeses were collected and studied for the prevalence of yeast species and the result cleared that 26 were positive to yeast isolation with the percentages of 52 %.

Yeast Species Isolated from Fresh Kareish Cheese: Based on traditional methods (sugar fermentation, assimilation culture on rice agar medium, tobacco agar medium and germ tube formation) for identification of different yeast species isolated from kareish cheese. The prevalence of yeast isolates were distributed as follow:

- 5*Candida albicans* (19.25 %), 5 *Geotrichum candidum* (19.25 %), 2 *C. glabrata* (7.7%), 2 *C. guilliermondii* (7.7%), 2 *C. tropicalis* (7.7%), one *C. dubliniensis* (3.85 %), one *C. krusei* (3.85 %), one *C. parapsilosis* (3.85 %), one *Cryptococcus neoformans* (3.85 %), one *Trichosporon cutaneum* (3.85 %) and mixed 5 yeast (19.15%) isolates were detected.

Biochemical Activities of Yeasts: Positive reaction of sugar fermentation indicated by change color of bromocresol purple to yellow color with gas or without gas and positive reaction of sugar assimilation indicated by growth of yeast colony around sugar disc. And positive reaction of urea hydrolysis indicated by change color of medium into pink color.

PCR was used for detecting phospholipase enzyme production as a rapid and more sensitive tool. As shown in Fig. (4). the forward and reverse primers produce a 751 bp DNA fragment.

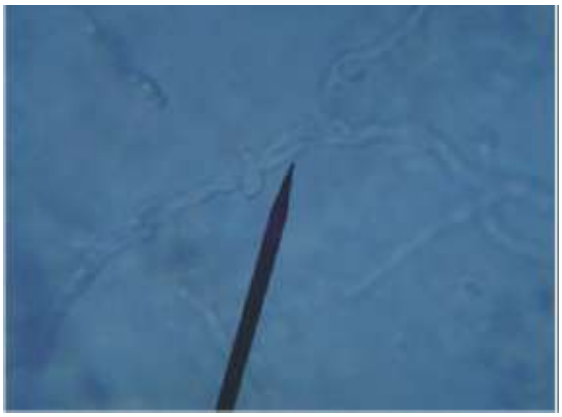

Fig. 1: Arthrospores formation of *Geotrichum candidum* on rice agar medium

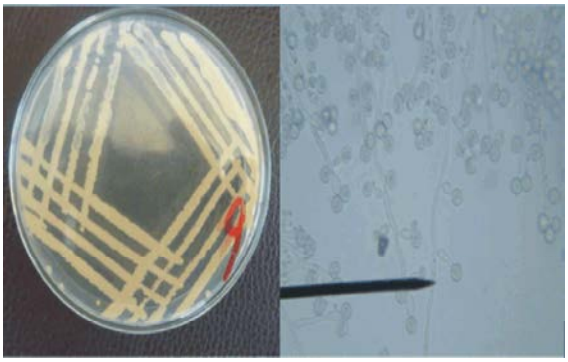

Fig. 2: Macroscopic and microscopic of *C. Albicans* on Sabouraud's dextrose (SAB)

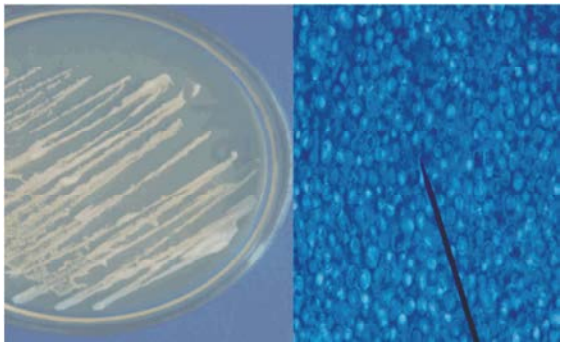

Fig. 3: Macroscopic and microscopic examination of *C. Neoformans* using india link stain: A halo of unstained capsule around the yeast cell

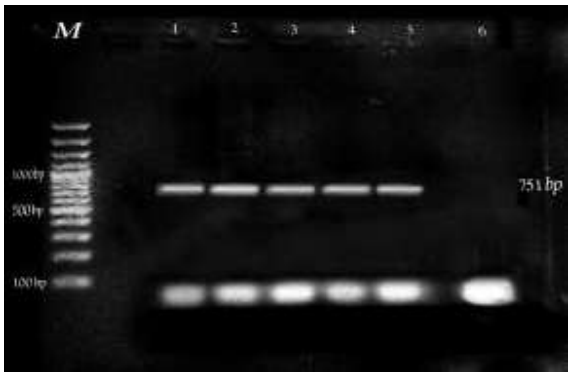

Fig. 4: Amplification of 751 bp by PCR for detection of phospholipase B (PLB1) gene. Lane M: 100 bp Ladder, lanes 1, 2, 3, 4 and 5 showing positive samples while lane 6 shows negative control result.

Detection of Lipase Gene of Geotrichum Candidum by PCR: PCR was used for detecting lipase enzyme production as a rapid and more sensitive tool. As shown in Fig. (5). the forward and reverse primers produce a 300 bp DNA fragment.

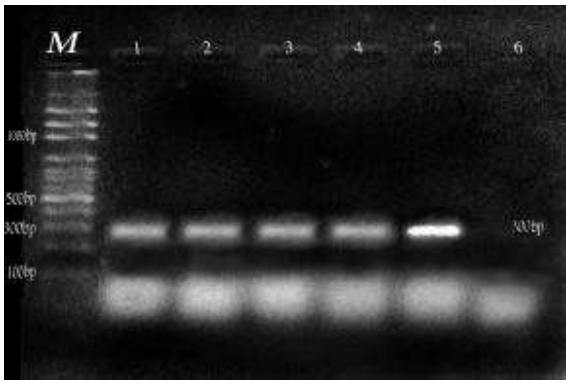

Fig. 5: Amplification of 300 bp by PCR for detection of lipase gene. Lane M: 100 bp Ladder, lanes 1,2,3,4 and 5 showing positive samples while lane 6 shows negative control result.

Table 1: Detection of yeast species percentages in kareish cheese

Yeast species	No of isolates	%
C. albicans	5	19.25
Geotrichum candidum	5	19.25
C. glabrata	2	7.7
C. guilliermondii	2	7.7
C. tropicalis	2	7.7
C. dubliniensis	1	3.85
C. krusei	1	3.85
C. parapsilosis	1	3.85
C. neoformans	1	3.85
Trichosporon cutaneum	1	3.85
Mixed yeast	5	19.15

% = the percentages were calculated according to the number of yeast isolates.

DISCUSSION

Results obtained in Table (1) showed that the most predominant yeast species isolated from kareish cheese were Candida spp. and *Geotrichum candidum.* Similar findings were reported by Krukowski [19] who found that all of the fungal isolates were yeasts of the genera *Candida, Rhodotorula* and *Trichosporon,* Pengov [20] also was in agreement with this result, noticed that the most prevalent yeast species were Candida spp. and Bourtzi [21] found that from the 42 fungi isolates, 38 were yeasts, belongs to the genera Candida, Geotrichum and Rhodotorula the results in Table (2) showed that sugar fermentation and assimilation were necessary and important tests for identification of yeast species, the results were confirmed by Finegold and Baron [22] stressed that biochemical identification is of great importance for laboratory identification of *C. albicans*. Wang [23] and Zaini *et al.* [24] concluded that Carbohydrate assimilation may be necessary for definitive identification of Candida species. These results in the same table revealed that *C. neoformans* was not fermentative and hydrolyzes urea. The results were also confirmed by Dorko *et al.* [25] and Viviani *et al.* [26]. Determination of phospholipase B gene as shown in figure (4) was detected by polymerase chain reaction (PCR) as a rapid and more sensitive tool for detection of this enzyme. Many authors like Ghannoum [27], Hoover *et al.* [28] and Sugiyama *et al.* [29] used PCR for detection of phospholipase activity. The secretion of extracellular phospholipases by *C. albicans* was first detected by Costa *et al.* [30] and Werner [31] by growing this yeast on solid media containing egg yolk and analyzing the lipid breakdown products. Although the literature contains contradictory reports on the number and speci?c types of phospholipase genes that may be linked to the virulence of *C. albicans* [32], the PLB1 gene appears thus far to be

Table 2: Biochemical activities of isolated yeast species

	Sugar fermentation				Sugar assimilation				
Types of yeast species	Glucose	galactose	Sucrose	maltose	Glucose	lactose	sucrose	maltose	Urease
C. albicans	+	+	-	+	+	-	+	+	-
C. dubliniensis	+	+	-	+	+	-	+	+	-
C. tropicalis	+	+	+	+	+	-	+	+	-
C. parapsilosis	+	-	-	-	+	-	+	+	-
C. guilliermondii	+	-	-	-	+	-	+	+	-
C. glabrata	+	-	-	-	+	-	-	-	-
C. krusei	+	-	-	-	+	-	-	-	+
C. neoformans	-	-	-	-	+	-	+	+	+
Geotrichum candidum	-	-	-	-	+	-	-	-	-

Detection of phospholipase B (PLB1) gene of C. albicans by PCR:

the single most important contributory factor for phospholipase activity of *C. albicans* [33, 34]. PCR has the ability to detect phospholipase enzyme producing gene in *C. albicans* strains even if it is produced in small amounts [35]. The results in Fig. (4) revealed that phospholipase was detected in all 5 tested *C. albicans* isolates.

On the other hand the secretion of extracellular lipases by *Geotrichum candidum* is rather unusual amongst soluble enzymes in that they exhibit increased activity at a water lipid interface [36].

Moreover lipase has also received increased attention recently in studies to investigate the molecular mechanism of its catalytic activity [37]. In the current study; for detection of lipase by PCR, The five morphologically and biochemically identified isolates of *Geotrichum candidum* were used and our results in Fig. (5) revealed that all five isolates give a positive result for presence of lipase specific band by PCR so these results agree with those of Rajesh *et al*. [38] and Sanio and Nilanjan, [18].

CONCLUSION

From the present work, it could be concluded that the high percentage of yeasts, particularly *C. albicans* and *Geotrichum candidum* isolated from fresh Kareish cheese revealed the great need for applying more hygienic measures during handling, processing of milk and manufacturing of cheese. The most reliable diagnostic measure is the accurate and rapid identification of *C. albicans* and *Geotrichum candidum* using polymerase chain reaction as the simplest molecular based technique. On the other hand the enzymes which may responsible for the virulence need more molecular characterization studies to detect their role in the yeast pathogenicity.

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