

Investigation of the Hydrolytic Enzyme Activities of *Candida Parapsilosis* Isolated From Milk Samples of Bovine Mastitis by API ZYM and Molecular Method

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Abstract

Two hundred and fifty milk samples were collected from cows suffering from mastitis, these samples were tested for mycotic mastitis using culture and conventional PCR method. The results showed 116 isolates of *Candida spp.* were recovered with a prevalence of (46.4%), hence *C. parapsilosis* was the dominating species 15/116 (12.9%). Fifteen isolates (100%) belong *C. parapsilosis* was confirmed by PCR using a species-specific primer for the 18S rRNA gene. Some randomly chose isolates of *C. parapsilosis* were used for detection of their hydrolytic enzymatic activity by API ZYM test and PCR. The method used to evaluate their enzymatic activity was API ZYM (BioMerieux). The highest activity was observed for leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α -glucosidase as 10/10 (100%) for each, followed by esterase lipase (C8) 9/10 (90%), valine arylamidase and alkaline phosphatase 8/10 (80%) for both and esterase (C4) 7/10 (70%). Conventional PCR was done for partial amplification of sterol esterase, alkaline phosphates and alpha-glucosidase genes by specific primer sequences, The results of the PCR amplification of these genes was 8/10 (80%) for sterol esterase gene. Concerning the alkaline phosphatase gene, it was present in 10/10 (100%). Regarding alpha-glucosidase gene was present in 10/10 (100%). This work aims at evaluating enzymatic activities of *C. parapsilosis* isolated from cow's milk with mastitis.

Keywords: *Candida parapsilosis*, Bovine mastitis, Hydrolytic enzymes, Conventional PCR, API ZYM test.

Introduction

Bovine mastitis is the most devastating state of the epidemic in terms of the worldwide economic losses. The etiological agents can vary from place to place depending on climate, animal species and animal husbandry, and include a wide variety of gram-positive and gram-negative bacteria and fungi⁽¹⁾. Mycotic mastitis had been documented to be caused by various genera of moulds and yeasts beside bacterial mastitis,

The most commonly observed fungi are *Candida spp.*, *Aspergillus spp.*, *Trichosporon spp.*, *Cryptococcus spp.*⁽²⁾. Mycotic mastitis is classified into two large groups based on the moment of diagnosis, primary mycotic mastitis (bacterial preliminary mastitis) and secondary mycotic mastitis that often occur immediately without antibiotic treatment⁽³⁾. For timely, effective management, early, rapid and accurate identification of the pathogenic fungus is important. In the clinical microbiology laboratory, traditional detection of pathogenic fungi is based on morphological and physiological tests that often take 3 days or more and may be inaccurate. A traditional PCR and multiplex PCR approach was developed in recent years to classify multiple fungal pathogens simultaneously in a single reaction⁽⁴⁾. The fungi have virulence factors such as adapted cell morphology, adhesion factors, phenotypic switching, biofilm formation and enzymatic activity to

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colonize, invade and evade host defence mechanisms. One particularly effective factor is the production of extracellular hydrolytic enzymes which facilitate the destruction of the cellular membranes and thus allow the host tissue to penetrate the fungal cells⁽⁵⁾.

Material and Method

Sample Collection: Two hundred and fifty samples of bovine milk with mastitis according to California mastitis test from different areas of Basrah province, had been collected during the period from March 2018 up to September 2019.

Isolation and identification of *Candida spp.*: Milk samples were inoculated in Sabouraud's Dextrose agar supplemented with 0.05 mg/ml chloramphenicol and then incubated at 37°C for 24h up to 1 week. Macroscopic and microscopic morphology tests have been performed after incubation yeast identification was conducted according to⁽⁶⁾.

Detection of hydrolytic enzymes by API ZYM Kit: The enzymatic activity was measured using a semi-quantitative micromethod API ZYM test (BioMerieux, France) (API Laboratories and Analytab products). The substrate was available for 19 hydrolytic enzymes⁽⁷⁾, for evaluating the followings: Alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol – AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucosidase, α -glucosidase, α -mannosidase and α -fucosidase.

Specimen suspension was expected from culture media on Sabouraud's dextrose agar in 2 ml of distilled water, then suspended in API medium the turbidity was concordant with McFarland tube No. 5. Inoculation of the strip using a pipette was done by dispensing 65 ml specimen into each cupule, the plastic lid was placed on the tray and incubated at 4 h for 37°C, the inoculated strip was not placed in bright light. One reduction of ZYM A, after incubation (tensioactive agent) and 1 reduction of reactive ZYM B (diazonium salt) was added to every cupule. Strips had been placed under a strong source of light (1000 W bulb) for about 10 seconds. Reactions were read and the results recorded on the result sheet. A value of 0-5 assigned corresponding to the established colours: 0 corresponds to a negative reaction; 5 corresponds to the maximum intensity; values 1, 2, 3, 4 are considered to be intermediate reactions depending on the intensity level.

Molecular identification of *C. parapsilosis*:

A. Primer Design: DNA extraction yeast culture was done with G-spin extraction DNA kit according to the manufacturer protocol identification of *C. parapsilosis* isolates were conducted by using conventional PCR for the amplification of a partial gene of 18S rRNA by specific primer sequences was used. The hydrolytic enzymes detected were of genes encoding sterol esterase, alkaline phosphatase and alpha-glucosidase. The PCR primers were designed and funded by the company (Macrogen, South Korea) using the NCBI- Gene Bank online database and primer design., table (1).

Table (1): Oligonucleotide primers used for 18S rRNA, sterol esterase, alkaline phosphatase and alpha-glucosidase genes in *Candida parapsilosis*.

No.	Gene (systematic name) primer	Oligonucleotide Primer sequence (3'-5') Forward & reverse		Amplicon (bp)	Genbank Accession number
1	18S rRNA	F	CTGCGGAAGGATCATTACAGA	507	FM172980
		R	TCCTCCGCTTATTGATATGCTT		
2	Sterol esterase	F	TGTGCCTCGAGAACCATACG	489	HE605208
		R	CTCTGGAGTCCACCTTGACG		
3	Alkaline phosphatase	F	GGGGCCACTGCATTTTCTTG	598	NC032089
		R	CATTGTGGTGTGAAGCGTGG		
3	Alpha-glucosidase	F	ATGCTACTCATGCCGATGGG	550	NC032089
		R	GTATCAACGCCGCAATTC		

B. DNA extraction: According to the manufacturer procedure, yeast genomic DNA from the milk samples was extracted using G-spin DNA extraction kit. *Candida* culture as 200 µl was pipetted into 1.5 ml sterile microcentrifuge tubes, then added 50 mg glass beads with 20 µl of proteinase K and homogenized for 5 min by cell disruptor vortex mixer. Then 200 µl of BL cell lysis buffer was added to each tube and mixed with a vortex mixer, all tubes were incubated for 10 min for 56°C with mixing every 3 min, 200 µl of absolute ethanol was applied to lysate and immediately mixed by shaking vigorously. Spin column put in a 2 ml collection tube and transferred the mixture (including any precipitate) to the column, and then centrifuged for 1 min at 13,000 rpm. The filtrate was discarded and the spin column was inserted into a new 2 ml collection tube, 400 µl W1 buffer was added to the spin column and centrifuged at 13,000 rpm for 1 min, the flow-through was discarded and the collection tube was reused. Six hundred µl wash buffer was applied to each column and centrifuged at 13,000 rpm for 1 min, the flow-through was removed, the column was put in a new 2 ml collection tube, then all the tubes were centrifuged at 13,000 rpm for 1 min to dry the column membrane. Spin column was placed in a new 1.5 ml tube, and 50 µl of the CE buffer was added directly to the membrane and incubated at room temperature for 1 min, then centrifuged at 13,000 rpm for 1 min. Instead all the tubes left stand for 1 min to ensure that the matrix absorbed the elution buffer, then centrifuged at 10,000 rpm for 30 seconds to elute the purified DNA. NanoDrop spectrophotometer was used to check the concentration of the extracted DNA according to the formula: 1OD260 = 50ng, purity = 260/280.

C. PCR amplification: PCR master mix reaction by using (Master mix PCR PreMix kit). The master mix was prepared according to company instructions by inserting 3 µl of template DNA, 1 µl of forward and reverse primers (10 pmol), and 20 µl of nuclease-free

water was completed. Reactions were as follows: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 18S rRNA, 60.3°C for sterol esterase, 59.3°C for each alkaline phosphatase and alpha-glucosidase for 30 sec, and extension at 72°C for 50-60 sec, with a final extension at 72°C for 5 min. Amplification products were separated by electrophoresis on 1.5 % agarose gel containing 3 µg/mL ethidium bromide using a 2000-bp ladder (Amersham Biosciences) as molecular weight marker. Tested on agarose gel electrophoresis for 1 hour (100V).

Results

- 1. Prevalence of *C. albicans* in dairy cows with mastitis:** Out of 250 milk samples analyzed from cows with mastitis, 116 *Candida* isolates were recovered (46.4%). Based on cultural and morphological aspects. *C. parapsilosis* was the predominant 15/116 (12.9%).
- 2. API- ZYM enzymatic activities:** Some random selected *C. parapsilosis* isolates were used for determination of 19 hydrolases activities. Regarding *C. parapsilosis* 10 isolates were generated highly activated leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and α-glucosidase when the percentage was (100%), followed by esterase lipase nine isolates produce this enzyme strongly (90%) meanwhile only 1 isolate (10%) showed no activity of this enzyme. Eight isolates (80%) found to be producers for each of alkaline phosphatase and valine arylamidase with high activity and only 2 isolates (20%) produce alkaline phosphatase with moderate activity, while 2 isolates (20%) with no activity for valine arylamidase enzyme. In case of esterase, it was found that 7 isolates only (70%) produce this enzyme with strong activity, meanwhile, 2 isolates only (20%) produce this enzyme in moderate activity and only 1 isolate (10%) showed no activity at all, table (2).

Table (2): Evaluation of the enzymatic activity of *C. parapsilosis* (N=10).

No	Enzyme	Strong activity		Moderate activity		Negative activity	
		No of isolates	%	No of isolates	%	No of isolates	%
1	Control						
2	Alkaline phosphatase	8	80	2	20		
3	Esterase (C4)	7	70	2	20	1	10

No	Enzyme	Strong activity		Moderate activity		Negative activity	
		No of isolates	%	No of isolates	%	No of isolates	%
4	Esterase lipase (C8)	9	90			1	10
5	Lipase (C14)						
6	Leucine arylamidase	10	100				
7	Valine arylamidase	8	80			2	20
8	Cystine arylamidase						
9	Trypsin						
10	α -chymotrypsin						
11	Acid phosphatase	10	100				
12	Naphtol-AS-BI-phosphohydrolase	10	100				
13	α -galactosidase						
14	β -galactosidase						
15	β -glucuronidase						
16	α -glucosidase	10	100				
17	β -glucosidase						
18	N-acetylo- β -glucosyloaminidase						
19	α -mannosidase						
20	α -fucosidase						

Enzymatic activity expressed in nanomoles A- Control no colour B- Strong activity represented in the level of intensity 5 C- Moderate activity in the level intensity of 1,2,3,4 D- Negative reaction corresponding 0.

3. Molecular identification of *C. parapsilosis*:

Molecular identification of *C. parapsilosis* in this study relied on conventional PCR for the amplification of a partial gene of 18S rRNA by specific primer sequences. This gene was present

in fifteen samples of *C. parapsilosis* with PCR product size of 507 bp, as shown in figure (1). Using NanoDrop spectrophotometer, DNA concentration was ranging from (5.9 – 70.2) ng/ml with a purity of (1.8–2.08).

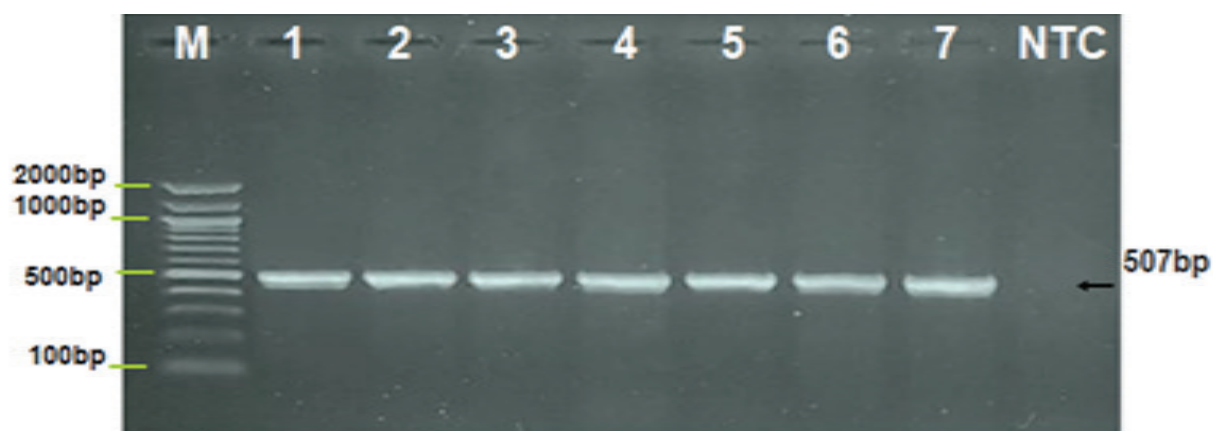


Figure (1): Agarose gel electrophoresis shows PCR product analysis of pathogenic *C. parapsilosis*. Lane M Marker ladder (2000 bp), lane (1-7): 18S rRNA gene of *C. parapsilosis* isolate at 507 bp. Lane (NTC): None template (negative control).

3. Conventional PCR assay for enzyme production by *C.parapsilosis*: The results of PCR amplification of these genes was 8/10 (80%) for sterol esterase with a PCR product size of approximately 489 bp, as shown in figure (2). Concerning the alkaline phosphatase gene, it was present in 10/10 (100%)

with a PCR product size of approximately 598 bp, as shown in figure (3). Regarding the alpha-glucosidase gene, it was present in 10/10 (100%) with a PCR product size of approximated 550 bp, as shown in figure (4).

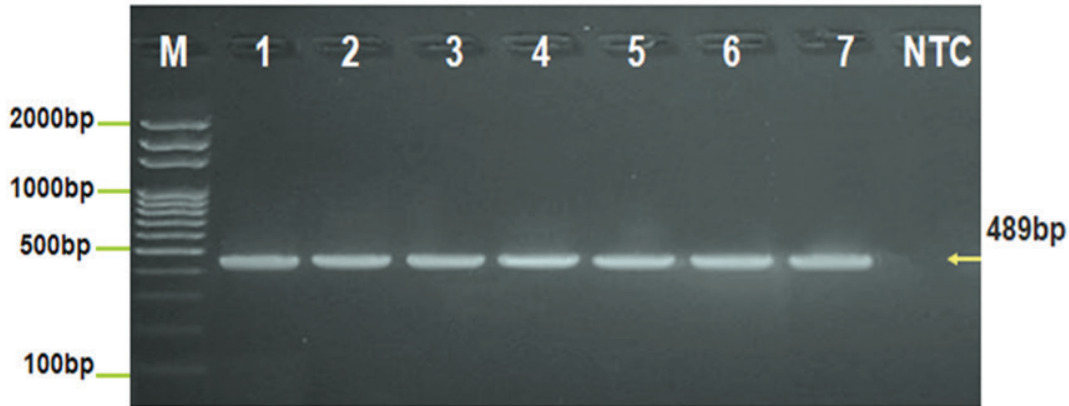


Figure (2): Agarose gel electrophoresis image shows PCR product analysis of pathogenic *C.parapsilosis*. Lane M Marker ladder (2000 bp), lane (1-7): Sterol esterase gene of *C.parapsilosis* isolate at 489 bp. Lane (NTC): None template (negative control).

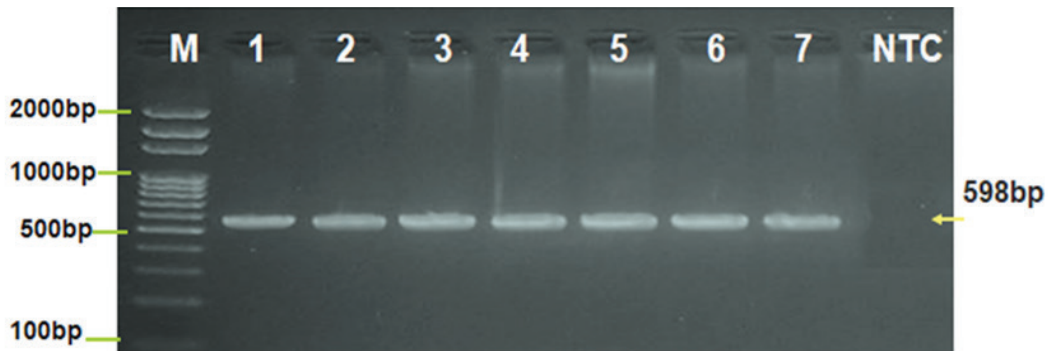


Figure (3): Agarose gel electrophoresis image shows PCR product analysis of pathogenic *C. parapsilosis*. Lane M Marker ladder (2000 bp), lane (1-7): Alkaline phosphatase gene of *C.parapsilosis* isolate at 598 bp. Lane (NTC): None template (negative control).

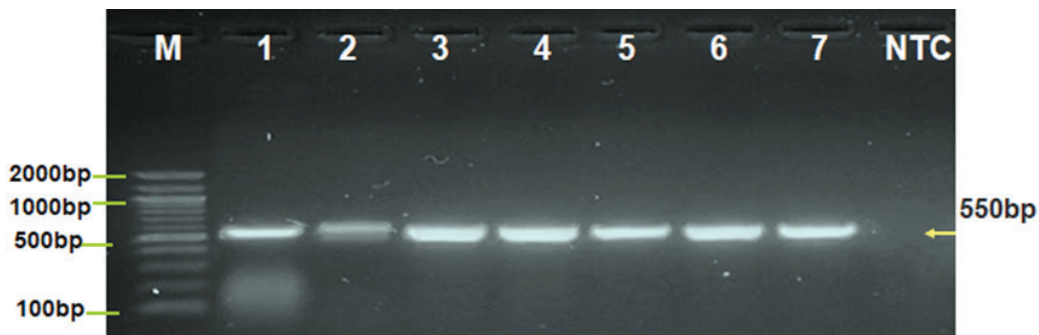


Figure (4): Agarose gel electrophoresis image shows PCR product analysis of pathogenic *C.parapsilosis*. Lane M Marker ladder (2000 bp), lane (1-7): Alpha-glucosidase gene of *C. parapsilosis* isolate at 550 bp. Lane (NTC): None template (negative control).

Discussion

Yeasts are single-celled organisms that are ubiquitous in the environment and they are considered prospective pathogens of the mammary gland that produce disease when natural defense mechanism is lowered^(8 and 9).

A total of 116 (46.4%) *Candida* isolates out of 250 milk samples were taken from cows with mastitis based on cultural, morphological aspects were carried out in various setting phenotyping identification tests for *Candida spp*. The highest percentage among isolated *Candida spp* was belong *C.parapsilosis* 15/116 (12.9%). In the present study, the percentage of *C.parapsilosis* was higher than those obtained in a study done in India (7.69%) isolated from cows with clinical mastitis⁽¹⁰⁾. The presence of *C. parapsilosis* were also incompatible with those reported in Poland which was (45%) and in Turkey as (12.7%)^(11 and 12). The geographical variation may be considered one of the reasons for the discrepancy in the distribution of species, or due to the number of included samples or may due to the differences in the method used for diagnosis.

In the present study *C.parapsilosis* (N=10) isolates and regarding hydrolases API ZYM test showed strong leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α -glucosidase among 10/10 (100%), esterase lipase (C8) 9/10 (90%), valine arylamidase and alkaline phosphatase 8/10 (80%), and esterase (C4) in only 7/10 (70%). Hydrolytic leucine arylamidase enzymes was produced strongly in both tested species of *Candida* which similar to those reported in Poland by⁽⁷⁾. And the isolates of *C. parapsilosis* examined did not produce trypsin, chymotrypsin, α -galactosidase, β -glucuronidase, α -mannosidase, and α -fucosidase which is similar to those reported by⁽¹³⁾. In the current results and regarding the enzymatic activity of the isolated *Candida spp*. showed some differences from those detected by⁽¹⁴⁾, who studied 12 enzymes of trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, N-acetyl-glucosaminidase and α -mannosidase. The highest activity was reported in alkaline phosphatase, leucine arylamidase, phosphatase acid, and phosphohydrolase naphthol-AS-BI. The disagreement of the current study from others may due to the geographical variations that affect the distribution of species hence affected their enzyme production ability.

Conventional PCR was done for partial amplification of sterol esterase, alkaline phosphates and

alpha-glucosidase genes by specific primer sequences. The results of the PCR amplification of these genes was 8/10 (80%) for sterol esterase. Concerning alkaline phosphatase, it was present in 10/10 (100%). Regarding alpha-glucosidase, it was present in 10/10 (100%).

In the current study the reasons of choosing these three enzymes out of the previously 19 studied ones is that the gene sequence of the other 16 enzymes are not found in gene bank i.e. the sequences of the three studied enzymes were obtained only. Furthermore, and concerning other studies in this field were not found as published research to compare the current results with them.

Conclusion

1. This study proved the presence of *C. parapsilosis* in cow milk sample with mastitis in Basrah province.
2. API ZYM test is a good and more specific test than conventional PCR for detection of hydrolytic enzymes activities

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq.

Conflict of Interest: Non

Funding: Self-funding

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