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Real time PCR for identification of Viable But Non Culturable fungi isolated from mastitic cattle milk samples depending on propidium mono azide stain

Hussein, A. M.[1] , Salem, R. M.[2] , El-Diasty,E.M.[2], and Marwa, A. H. [2]

(1) Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Sadat City University.
(2) Mycology Department, Animal Health Research Institute, Dokki, Egypt.
*** Corresponding Author:** marwahamoudao@gmail.com ***Submitted:*** *1 Sept. 2019* ***Accepted:*** *12 Sept. 2019*

ABSRACT

A total of 150 milk samples were collected from both acute and chronically infected cattle from both Giza and Minoufia governorates. Incidence of mycotic mastitis was at rate of 72% and 88%for Giza and Minoufia governorates respectively. Different mold and yeast species were isolated. The most isolated mold species from Giza samples were Aspergillus species (64.4%) followed by Geotrichum (15.2%)then Penicillium (10.2%), while in Minoufia governorate, the most isolated mold species were Penicillium at a percentage of (50.6%)followed by Aspergillus at a percentage of (40.2%). About yeast species isolation, *C. parapsillosisis* was the most isolated yeast species from Giza samples with percentage of 33.4%,while in Minoufia samples, *C. tropicalis* was the most isolated yeast species with a percentage of 22.7%. All of the isolates are identified depending on macroscopical and microscopical identification. Twelve isolated yeast strains were biochemically identified depending on rapaid yeast plus identification system, all of the tested strains were correctly identified except for *C. parapsillosis* strains, only 83%of the tested strains were correctly identified. Six negative examined milk samples with culture on Sabaroud dextrose agar media were subjected to Rt-PCR and Propidium mono azide stain, four of them were positive although they were negative on culture as they are considered as samples containing VBNC(Viable but non culturable) strains.

Key word: *Fungi, Mastitis, Cattle, Rt-PCR, Propidium monoazide*

INTRODUCTION

Bovine mastitis is a disease caused by a wide variety of microorganisms that causes large economical losses and damages to the dairy industry by decreasing milk production and through increasing costs of antibiotic treatment and culling of the diseased animals (Zaragoza *et al*., 2011)

Some yeasts and molds able to induce the condition of mycotic mastitis. Mastitis due to filamentous fungi mostly *Aspergillus fumigatus* has been reported. It occurs as sporadic cases affecting a small percentage of cows or as outbreaks affecting the majority of animals (Abd El Razik *et al*., 2011)

Bovine mycotic mastitis is usually caused by yeasts. The most frequently isolated yeasts from milk are *Cryptococcus neoformans* and *candida albicans* due to prolonged and indiscriminate use of antibiotic and steroids in intra-mammary therapy for mastitis as well as prevalence of fungal organisms on dairy farms (Rayaz and Darand, 2013).

Identification of those pathogenic fungi is based on morphological and physiological tests

often require 3 or more days and may be inaccurate (Luo and Mitchell 2002)

Culture methods only allow counting of viable cells that are capable of forming colonies on the media, without detecting dead cells, viable but non-culturable cells (VBNC) and those that require special growth conditions (Cerca *et al*., 2011), on the other hand, qPCR detects all cells in a sample, including the dead cells or the DNA of some of them that can be found in the environment (Pathak *et al*. 2012)

In recent years, a new intercalating agent has been used together with qPCR reaction to discriminate and count both live and dead cells in a microbiological sample. This new methodology is based on the use of propidium monoazide (PMA).

The present study aimed to identify and characterize of the causative agents of mycotic mastitis in cattle with traditional methods and biochemical identification with RapID system with detection of viable but non culturable fungi with real time PCR and propidium mono azide stain.

The aim was achieved by isolation and identification of fungi from mastitic milk samples by traditional methods, biochemical identification of some yeast isolates with RapID Yeast Plus System, and Identification of viable but non cultrable organisms with real time PCR and propidium mono azide stain .

MATERIALS AND METHODS

Collection of samples:

A total of 150 milk samples,75 from Giza and 75 from Minoufia governorates in Egypt were hygienically collected from dairy cattles with clinical and subclinical mastitis.

Isolation and identification of mold from milk samples: (NMC,1999)

A loopful from the sediment of the centrifuged milk samples were streaked onto the surface of Sabaroud's dextrose agar plates. Half Plates were incubated aerobically at 37°C and the other at 25°C for 5-7days.

The developing fungi were identified according to macro and microscopic characteristics as described by (Pitt and Hocking, 2009).

Identification of yeast depending on RapID yeast Plus System: (Salkin *et al*., 1987)

Suspend sufficient of growth colony in inoculation fluid 2ml tube and continue add colonies to achieve a visual turbidity. Distribute the inoculation fluid in the panel, Incubate for 4hours in a non co_2 incubator at 30 ºC, Add the Provided Reagent (internal reagent A), 1 drops- to the wells no. 7 through 14. Add the Provided Reagent (internal reagent B), 1 drops- to the wells no. 16 through 18, allow at least 30 seconds but no more than 1 minute for color development. Read color change in all panel (compare with Color Guide and scor -ve and +ve results in report form).

Molecular identification depending on RT-PCR and propidium mono azide stain

Molecular identification was performed according to **(Mirhendi *et al*., 2007)**

-DNA was extracted according to QIAamp DNA Mini Kit. Catalogue no.51304.

-Quantitect SYBR green PCR kit Cat. No. 204141 containing 1 ml 2x QuantiTect SYBR Green PCR Master Mix, 2 ml RNase-Free Water. used for master mix preparation for SYBR Green real time PCR.

The PMA stock solution was transferred to 500 µl culture mixtures at a final concentration of 100 µM. All manipulations of PMA solution were performed under minimal light to prevent any potential chemical change in PMA structure. Following 10 min of incubation in the dark, samples were exposed for 5 min to a 500-W halogen light source at a distance of 15 to 20 cm from the light source. Tubes were placed on ice during the light exposure to avoid excessive heating.

Table (1): Oligonucleotide primers sequences source: Metabion (Germany).

Gene	Primer	Sequence	Reference
ITS	ITS.1	TCC GTA GGT GAA CCT GCG G	**Mirhendi *et al*., 2007**
	ITS.4	TCC TCC GCT TAT TGA TAT GC	

Methods for propidium iodide staining according to (Taskin et al.., 2011)

RESULTS AND DISCUSSION

Out of 75 examined samples from Minoufia governorate 88% were positive for fungal isolation and 72% of the 75 examined samples from Giza governorate as shown in table 2. This result agree to a wide extent with (Pachauri *et al*., 2013) who found 64 samples out of 100 samples (64%) were positive for fungal isolation, while those results are to some extent far from results of (Khalid *et al*., 2011) who showed that, the overall positive percentage of fungal isolates was 25.2 % out of 123 examined milk samples.

Aspergillus and *Geotrichum candidum* isolated from Giza governorates were the same isolated mold species by (Samir *et al*., 2015) from his examined milk samples except for *Aspergillus amstelodami* (from glaucus group), which we haven't isolated from our examined samples and instead, we have isolated two mold species did not isolated by (Samir *et al*.,2015) those spp. were , Fusarium and Penicillium. (Table 3)

C. parapsillosis was the most predominant isolated species from masitic milk samples collected from Giza governorate, while Rhodotorula and Saccharomyces were the least isolated species. In our study , *Cryptococcus neoformance* not detected, we agree with (Esraa *et al*., 2015;Bakr *et al*., 2015) as, *Cryptococcus neoformans* not detected in any examined sample of them, but we don't agree with (Abou Elmagd *et al*., 2011) who isolated *Cryptococcus neoformans* from milk samples collected from animals from Quasna governorate. However we don't agree with (Esraa *et al*., 2015) in Candida spp. isolation as *Candidaalbicans* took the lead of the isolates by 60 %, while in our study, the chief isolated candida species were *C.parapsillosis* 33.4% **.**

Results in table (5) showed that Penicillium was the most predominant isolated mold genera from samples collected from Minoufia governorate, followed by Aspergillus and Geotrichum, while Alternaria and Mucor were the least isolated mold genera with equal percentage of1.5 % . All of those isolates were detected in the study of **(Samir *et al.,* 2015)** except for Penicillium spp.

C. tropicalis major isolated yeast strains from milk samples in Minoufia governorate, followed by *C. parapsillosis*, Rhodotorula, Saccharomyces, Torulopsis and Trichosporon respectively.While the least isolated strains were *C.kruseii, Pseudotropicalis* and Debaryomyces respectively. While C. albicans not detected the same as a study in southern Brazil that failed to detect *C. albicans* in the milk of animals with clinical and subclinical mastitis, even though the species of the genus Candida were 37.9 % of fungus isolates (Spanamberg *et al*., 2008).

The RapID Yeast Plus System is an accurate and reliable method for yeast identification alternative to other commonly used yeast identification systems, our results shown in table (7) reveal that the correctly identified strains range between 100% to 83%. those results agree to a great extent with (Jamshid *et al* .,1999) who documented that of 117 isolates tested by the RapID Yeast Plus System, 96.6% identified correctly and also this result is the same as (Michelle *et al*., 2016) who identified 95.3% correctly and those results agree to a great extent with our results. (Verweij *et al*., 1999) results are 76.9 % correctly identified isolates and those results also are in a near range to this study.

Six negative examined milk samples (negative by culturing on SDA media), three from Giza and three from Minoufia governorates were subjected to real time PCR using the universal ITS primer for fungi. Four of them were positive with Real time PCR even though, they were negative on the culture as shown in table (8) and figure(1), while the other two samples were negative with real time PCR.

Table (2): Incidence of fungal isolation from Minoufia and Giza governorates samples:

Location of samples	*Total No. of examined samples*	*+ve samples*		*-ve samples*	
Minoufia governorate	*75*	*No.*	*%*	*No.*	*%*
		66	*88*	*9*	*12*
Giza governorate	*75*	*54*	*72*	*21*	*28*

Table (3): Isolated mold species from milk samples collected from Giza governorate

Mold species	No	%	Clinical samples	Subclinical samples
Aspergillus spp.	38	64.4	23	15
Fusarium spp.	4	6.8	4	--
Geotrichum candidum	9	15.2	2	7
Monelia spp.	2	3.4	2	
Penicillium spp.	6	10.2	2	4
Total	57	100	31	26

Table (4): Isolated yeast species from milk samples collected from Giza governorate

Yeast species	No	%	Clinical samples	Sub clinical samples
Rhodotorula	4	22.2	4	-
C. parapsillosis	6	33.4	4	2
C. krusei	2	11.1	2	-
Torulopsis	4	22.2		4
Saccharomyces	2	11.1	2	-
Total	18	100	14	8

Table (5) Isolated mold genera from milk samples collected from Minoufia governorate.

Mold species	No	%	Clinical samples	Sub clinical samples
Alternaria alternaria	1	1.5	1	--
Aspergillus spp.	26	40.2	25	1
Geotrichum candidum	4	6.2	4	--
Mucor	1	1.5	1	--
Penicillium spp.	33	50.6	33	--
Total	65	100	64	1

Table (6): Isolated yeast species from milk samples collected from Minoufia governorate:

Yeast species	No	%	Clinical samples	Sub clinical samples
Candida species				
C.kruseii	2	4.5	2	--
C. parapsillosis	8	18.2	8	--
C.pseudotropicalis	2	4.5	2	--
C.tropicalis	10	22.7	10	--
Debromyces	1	2.4	1	--
Rhodotorula	7	15.9	7	--
Saccharomyces	6	13.6	6	--
Torulopsis	5	11.4	5	--
Trichosporon	3	6.8	3	--
Total	44			

Table (7) Comparison between Phenotypic and RapID yeast plus system identification of some isolated strains

Strain	Phenotypic identification	RapID yeast plus system identification	Percentage correctly identified
C. parapsillosis	6	5	83%
C. tropicalis	2	2	100%
Geotrichum candidum	2	2	100%
Saccharomyces Cervisiae	1	1	100%
Trichosporon beigelli	1	1	100%

Table (8): Real time PCR for six milk samples negative on SDA.

Sample	ITS SYBR green rt-PCR
1	+
2	-
3	-
4	+
5	+
6	+

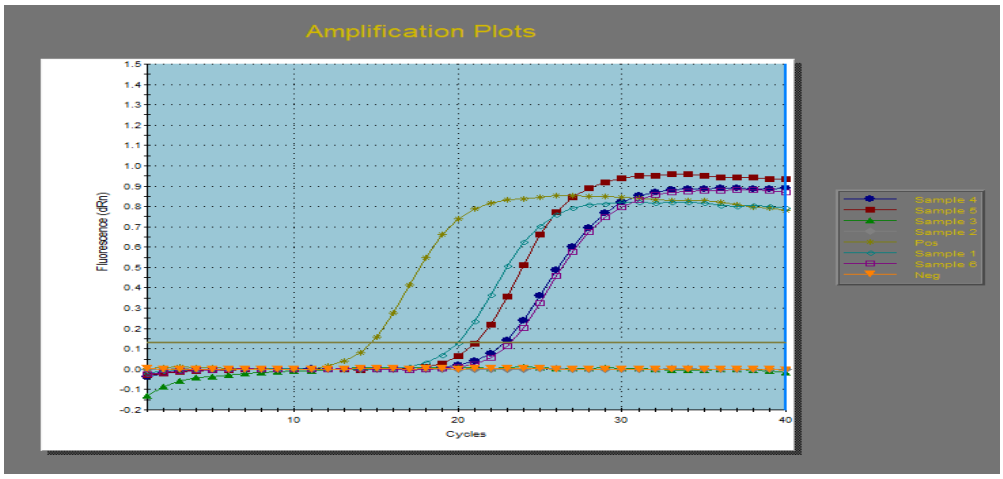

Fig. (1) The amplification plots of the Real time PCR for six negative milk samples on the culture (Sabaroud dextrose agar).

Positive samples with ITS SYBR green rt-PCR (sample1,4,5,6) were subjected to propidium mono azide dye that penetrates the porous membranes of dead cells but not intact membranes of live cells. so after propidium treatment to the four positive samples with rt-PCR, only one sample (sample 4) was positive with rt-PCR and this indicates that this is the only sample containing VBNC microorganisms and the other three samples containing dead cells. as detected in **table (9) figure (2)**.

Table (9): SYBR Green real time PCR after propidium mono azide exposure.

Sample after propidium treatment	**Fungal ITS**
1	-
4	+
5	-
6	-

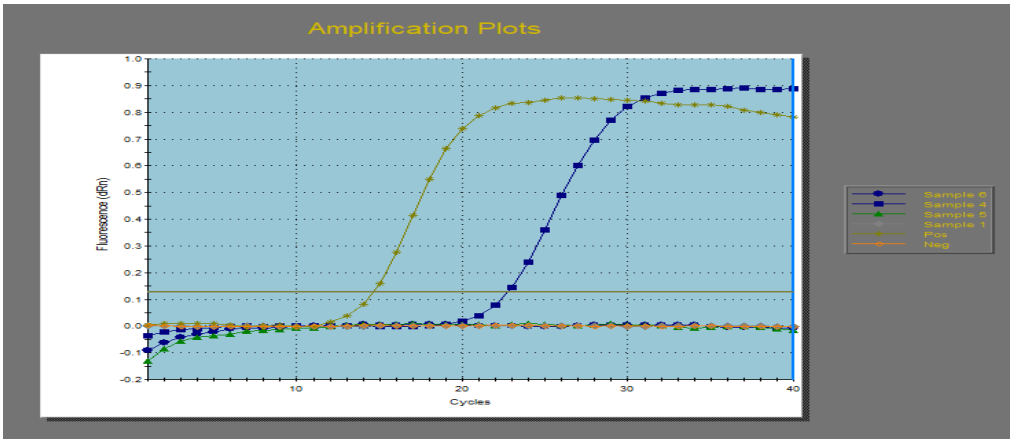

Fig. (2) Amplification plots of four milk samples after propidium treatment**.**

CONCLUSION

Mycotic mastitis is one of the most important diseases not only due to the cost of treatment but also due to the high culling rate. The results of the present study suggest that Candida spp. and Aspergillus spp. are the main fungi involved in bovine mastitis in both Giza and Minoufia governorates. Macroscopic and microscopical identification of different mold and yeast species is time consuming, so identification of yeast depending on biochemical identification kits is better as the results can be obtained within hours. Culture methods only allow counting of viable cells that are capable of forming colonies on the media without detecting dead cells, viable but non-culturable cells (VBNC) and those that require special growth conditions. On the other hand, qPCR detects all cells in a sample, including the dead cells or the DNA of some of them that can be found in the environment with the aid of propidium mono azide stain .

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