

Biochemical Studies on the Virulence Factors of Fungi Associated with She-Camel Milk

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ABSTRACT

Milk is an ideal habitat for the growth and multiplication of microorganisms due to its nutritional constitution which contains protein, carbohydrate, mineral and vitamins. All these components support the growth of many forms of bacteria, fungi and yeasts. Raw milk aseptically drawn from a healthy animal usually contains a few bacteria. This work aimed firstly to study the virulent factors of isolated yeasts in raw camel milk, and secondary detection the efficacy of PCR as a method of detection of the yeast virulence. A total of 40 samples were collected from camel milk from different localities in Egypt and were subjected for subclinical mastitis "California mastitis test". Results revealed that 4 samples (10%) have subclinical mastitis and rest of samples was negative to the test. From the results of fungal examination and isolation on specific media, we found that 6 samples were single infection (15%) and 2 were mixed infection (5%). Identification of these isolates were *Aspergillus Niger* 1 (2.5%) *Aspergillus fumigatus* 1 (2.5%), *Rhizopus* 1 (2.5%), *Mucor* 1 (2.5%), *penicillium* 2 isolates (5%), and *Candida albicans* 4 isolates (10%). We found that all *C. albicans* have ability to produce germ tube by incubation on rabbit serum, Chlamydo-spore, and phospholipase enzyme by using PCR to detect this enzyme as a rapid and sensitive method, so we found that all isolates have ability to produce that enzyme and we can use PCR also as a direct method for Identification with high efficacy. From the results, it can be concluded that low incidence of fungal agents associated with she-camel's milk and the main common Moulds and yeasts associated with mastitis were zygomycetes "*Rhizopus* 1 (2.5%), *Mucor* 1(2.5%)", *Aspergillus* "*Fumigatus* 1 (2.5)%, *Niger* 1 (2.5%), *Penicillium* 2 (5%)" and *Candida albicans* 4 (10%). Virulent factors of *C. albicans* germ tube, chlamydo-spores, Proteolytic and Phospholipase activities Which can be detected by using PCR.

KEY WORDS: Camel milk; Yeast; Mastitis.PCR.

INTRODUCTION

Camels (*Camelus dromedaries*) belong to the family Camelidae and the sub-order tylopoda. Sudan has the second largest number of camels in Africa. The population of camels in Sudan was estimated to be 2.8 million (FAO, 1990) distributed around the country. They belong to the one-humped dromedary kind, which originally reached the country from Arabia. The average daily milk yield per camel in Sudan was found to be 5 – 10 Kg (El-Amin, 1979). Nowadays, public health concern associated with microbial food safety has arisen. Numerous epidemiological reports have implicated non-heat treated milk and raw-milk products as the major factors responsible for illnesses caused by food-borne pathogens (Harrington *et al.*, 2002). Cross-contamination with pathogenic microorganisms can gain access to milk either by fecal contamination or by direct excretion from the udder into milk. Camel meat and milk are the key foods in arid and semi-arid areas of the African and Asian countries.

Camel milk has the ability to inhibit the growth of pathogenic microorganisms because it contains number of enzymes with anti-bacterial and anti-viral properties such as Lactoferrin which prevents microbial growth in the gut, Lactoperoxidase that suppresses Gram negative bacteria and most effective in raw milk during the first 4 days, peptidoglycan recognition protein (PGRP) that broad anti-microbial activity, stimulates the immune system, N-acetyl-

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glucosaminidase (NAGase) antiviral activity, Lysozyme which inhibits the growth of bacteria, and has effective influence on the storage camel milk, and immunoglobulins which possess several traits which give them tremendous advantage over conventional antibodies (Wernery, 2003).

Camel's milk not only contains more nutrients compared to cow milk, but also it has therapeutic and antimicrobial agents. In fact, most of camel milk was consumed in the raw state without any heat treatments or acid fermentation and the lack of refrigeration facilities during milking and transporting make the milk to be unsafe, and spoil fast (El-Ziney & Al-Turki, 2007).

Yeasts are widely distributed in nature and are therefore often found as contaminants in both commercial and traditional fermented milk (Gadaga *et al.*, 2000). Several researchers have reported yeast counts ranging between 10³–10⁷ log (CFU/mL) in fermented milk products (Viljoen *et al.*, 2003). Depending on the type of the fermented milk produced, their contribution may either be positive or negative. In yoghurt, their occurrence is mainly a consequence of the contamination and hence they are a major cause of yoghurt spoilage (Fleet, 1990). On the other hand, during the commercial production of kefir and koumiss they are deliberately introduced into milk to bring about the desired aroma and flavor of the final product (Wyder, 1998). In naturally fermented milk, yeasts are part of the indigenous microflora, coming into the product with the raw milk or from the environment and containers (Gadaga *et al.*, 2000).

Mastitis has both an extreme zoonotic and economic importance. It is the cause of multiple hazardous effects on human health and animal production. Camel's milk has characterized with a well-balanced composition suitable for human consumption, especially in deserts. It has relatively high content of vitamin C, whey proteins and non-protein nitrogen, and fat with higher long chain fatty acids such as palmitic and stearic acids (Mohamed, 2006).

Vidotto *et al.*, (1999) suggested that the correlation between phospholipase activity and high germ tube production in *C. albicans* strains could facilitate the penetration of yeast through tissue. Also, Naglik *et al.*, (2003) stated that extracellular proteolytic activity plays a central role in pathogenicity of candida through allowing successful colonization. Eman el- shahat, (2007) stated that proteinases, phospholipases secretion, hyphal formation and phenotypic switching of *C. albicans* were required for virulence. Where proteinase activity associated with tissue invasion. Ghannoum, (1998) have gene encoding protein using a PCR-based approach. Macura *et al.*, (1998) reported that 25 *C. albicans* strains were correlated with their genotypes as determined by PCR finger printing and the adherence capacity. The objectives of the present study are determining the biotechnological assays for Virulence factors of mould and yeasts associated with of camel milk.

MATERIALS AND METHODS

I- Camel milk samples:

A- Camel: A total number of 40 quarter milk samples were collected from she camels at different localities in Egypt. The udder of each camel was palpated before sampling for detecting any abnormalities such as swelling, asymmetry or any other physical changes. Each udder was washed and carefully dried with clean towel. The teats were then swabbed with 70% alcohol, the first jets of milk were rejected then 15 -20 ml of milk were drawn from each quarter into a sterile screw capped test tube. Some other milk samples for separation of lyophilized powder from camel milk serum and other as reserve if needed. The milk samples were kept in an ice container and forwarded to the laboratory for examination.

II. Materials used for California Mastitis Test (CMT):

Mastitis indicator test kit (Frieso-test): was obtained from Impfstoffwerk Friesoythe GmbH-Germany. California Mastitis Test (CMT) (APHA, 1992).

III- Media:

- 1- Media used for isolation of mould and yeasts was carried according to (Cruickshank *et al.*, 1975; Al-doory, 1980).
- 2- Media for complete identification: was prepared according to (Refai *et al.*, 1969; Lodder and Kreger, 1970; Feingold and Baron, 1986).
- 3- Detection of virulence factors of yeasts: determination of proteinase activity (Aoki *et al.*, 1990) and phospholipase production (Polak, 1992).
- 4- Detection of *C. albicans* DNA by PCR (Mohamed, 2002; Munoz *et al.*, 2003): Primers: (1 µm each) Determination of phospholipase B (PLBI) by polymerase chain reaction:
C. albicans was grown on SDA for 24 h. Fourty- five milliliters of 2% sabouraud broth was inoculated with one to three colonies of *C. albicans*. Cultures of *C. albicans* were grown for 18 h at 37 °C in a reciprocal shaking water bath set at 80 oscillation/min.

- The cultures were harvested by centrifugation for 10 minutes at 2.000 Xg. (Daman / IEC CRU-5000 centrifuge). The cells washed with normal saline repelleted and resuspended in 15 ml normal saline.

- DNA isolation:

- A 108 CFU suspension of *C. albicans* (200 ul) was mixed with 300 µl of lyses buffer. Samples were transferred to a homogenization tube containing beads and immediately processed in the FP 120 fast prep cell disrupter for 30 seconds. All samples were processed twice. Between processing the tubes were cooled on ice for 10 min. the samples were then centrifuged for 5 minutes at 14.000 Xg to pellet cell debris.

- Protoplast formation:

- Yeast spheroplasts for *C. albicans* were prepared by resuspending cells in 1.5 ml of protoplasts buffer. Pellets were resuspended by vortexing and then incubated for 45 min. at 30 °C. Upon completion; protoplasts were pelleted by centrifugation for 10 minutes at 2.000 xg.

- Each pellet was resuspended in 800 ul of LB. spheroplasts were then incubated for 30 minutes at 65°C. Tubes were once again centrifuged and the lysate was transferred to clean tube. Lysate was purified.

- Phenol chloroform purification:

- 200 ml phenol – chloroform – isoamyl alcohol (25:24: 1, vol, vol, vol) were added to lysate. Repeat mixing was done to it using vortex for 30 seconds and was centrifuged for 15 min at 14.000 rpm at 4 °C until a white interface was no longer present. Chloroform was added to remove any phenol residues.

- DNA precipitation:

- Purified lysates were transferred to a clean microcentrifuge tubes. To each tube 32 µl of 5 M sodium chloride was added followed by 2 volumes of 100% ethanol. DNA was precipitation for 1 hr at 20 °C and then pelleted at 4 °C in a microcentrifuge at 14.000 xg for 30 minutes. The supernatant was decanted, and the pellets were gently rinsed with ice- cold 70% ethanol. Pellets were dried in a vacuum desiccator with p2o2 to allow the ethanol to drain away. Dried DNA pellets were resuspended over night in 50 µl of TE. DNA samples were then treated with RNase (29 µg/ml) for 1 h at 37 °C for digestion of RNA which found after lysis of the cells and the purity of DNA solution was measured by running of 10 µl of each sample on a 1% agarose gel. Total chromosomal DNA isolated from the respective strain was subjected to PCR with oligonucleotide primers identical to the 751 bp region of the PLBI gene. Oligonucleotide primers (BioSynthesis) used in the PCR reactions was:

- Forward: 5'- ATGATTTGTCATCATTG-3'.

- Reverse: 5'- AGTATCTGGAGCTCTACC-3'.

5- Analysis of the PCR amplification products using agarose gel electrophoresis (Ibrahim, 1997):

RESULTS

Table (1): Prevalence of mastitis in quarter camel’s milk samples:

Total No. of camels	Sub clinical mastitis		Clinical mastitis		Normal samples	
	No.	%	No.	%	No.	%
40	4**	10	-	-	36	90

% :- Were calculated according to the total number of examined quarter milk samples.

Table (2): Number of single infection and mixed infection:

No. of samples	No. of single infection		No. of mixed infection	
	No.	%	No.	%
40	6	15	2	5

% :- Were calculated according to the total number of examined samples.

Table (3): Characterization of the isolated yeasts from camel’s milk:

No. of sample	No. of positive samples (Yeasts)		Type of the isolated yeasts		
	No.	%	Type	No.	%
40	4	10	C. albicans	4	100

% :- Were calculated according to the total number of examined samples.

Table (4): Characterization of the isolated moulds from she camel's milk:

No. of sample	No. of positive sample		Type of the isolated mould		
	No.	%	Type	No.	%
40	6	15	Rhizopus	1	2.5
			Mucor	1	2.5
			Penicillium	2	5
			Aspergillus niger	1	2.5
			Aspergillus Fumigatus	1	2.5

% :- Were calculated according to the total number of examined samples.

Table (5): Testing of *Candida albicans* isolates for virulence factors activity:-

Type of test		Type of yeasts			
		<i>Candida albicans</i> "4"			
		No (+ ve)	%	No (- ve)	%
1-	Phospholipase	4	100	0	0
2-	Urease	0	0	4	100
3-	Germ tube	4	100	0	0
4-	Chlamyospore formation	4	100	0	0

% :- Were calculated according to the total number of examined samples.

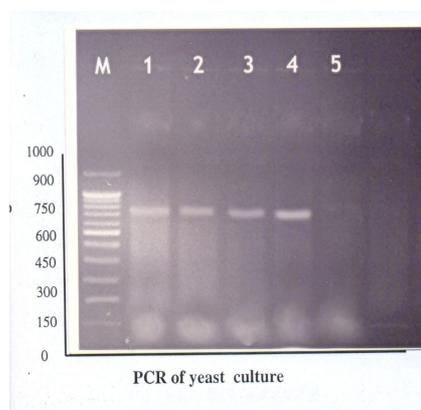


Figure (1): PCR of Yeast culture

DISCUSSION

Camel milk acquired a great importance as many trials explained. It's medical and antimicrobial effect as mentioned by (Elagamy *et al.*, 1992; Agrawal *et al.*, 2005). In this study during collection of camel milk samples, examination of udders was done to detect any abnormalities or clinical mastitis. There was no clinical mastitis in examined 10 she camels. This concluded that incidence of mastitis in she-camel is rare and this agree with Bolbol, (1982) and noted that mastitis in she-camels was rare in this species. California mastitis test (CMT) was applied to detect sub clinical mastitis. From 40 camel milk samples, there were 4 positive samples with a percentage of 10% and other 36 samples were negative table (1). The same results recorded by Sergeant *et al.*, (2001) and we noted that the prevalence of mycotic infection in milk samples was less than those reported by (Cuci and Matraku, 1987) (34%) and (Okamata *et al.*, 1988) 52%. And also, *Candida* species was the predominant yeast species and 4 isolates (100%) were isolated out of examination 4 isolates Pengov (2002). The result in table (5) the prevalence of moulds was 66.6% out of 8 fungal isolates, (Simaria and Dholakia 1986) stated that prevalence of mould, was (79.81%) out of 109 fungal isolates. In table (5), The most common isolates from milk samples were *Aspergillus Spp.*, *Mucor spp.*, *Penicillium spp.*, *Rhizopus spp.*. The obtained result nearly similar to those mentioned by (Chhabra *et al.*, 1998) which done on cow milk. Also the results abstained in table (5) revealed that penicillium spp was the most predominant mould isolates (33.3%). On the other hand, *Aspergillus niger* was the most common mould isolates (33.3%) reported by Chhabra *et al.*, (1998). Marcos *et al.*, (1990) discussed that *A. fumigatus* was the most common mould (37%) and Prabhakar *et al.*, (1989) stated that *mucor spp* was the most common mould (47.36%) which was done on cow milk. Also, in the present study, table (6) and (7) showed that sugar fermentation and assimilation tests for identification of yeast species as confirmed by (Wang, 1990) on cow milk samples.

Also, *C. albicans* isolates have the ability to produce different varieties of virulent factors that help it in adherence, pathogenesis, inducing the disease process in mammary glands. All *Candida spp.* produce chlamydospores and germ tube and these results revealed that formation of germ tube and chlamydospore formation is considered as confirmatory result for identification of *C. albicans* as recorded by (McCullaugh *et al.*, 1995; Enzenberger *et al.*, 1997). Silva and Paula (1998) depended on germ tube and chlamydospore formation. For identification of *C. albicans*. Also Zaini *et al.*, (2006) found all *C. albicans* isolates produces germ tube and chlamydospore. Hyphal formation (germ tube) of *C. albicans* was considered a role of pathogenesis of yeast as mentioned by (Hoppe and Frey, 1999; Yang, 2003). Also, the ability to produce phospholipase activity was (100%) for *C. albicans* isolates (Birinci *et al.*, 2005).

Determination of phospholipase production was detected also by polymerase chain reaction (PCR) as a rapid and a more sensitive tool for the detection of this enzyme as it detects the enzyme inspite of the variation of the precipitation zone that detected in egg yolk media which produced poor phospholipase- producing fungal isolates. The results showed that PCR has the ability to detect phospholipase ability in *C. albicans* strain even if it is produced in small amounts where it detected phospholipase production in all *C. albicans* strain.

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