MOLECULAR DETECTION OF STAPHYLOCOCCUS AUREUS ENTEROTOXINS ISOLATED FROM MASTITIC SHE-CAMELS

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ABSTRACT

A total of 150 raw milk samples were collected from she camels from Aswan, 100 from individual breed in Daraw and 50 from pastoral camels in Shalateen in Egypt. First, clinical examination revealed no cases with clinical mastitis. Second, the collected milk samples were tested by California Mastitis Test and the results revealed that the percent of subclinical mastitis was (39.3%) for total number of milk samples, but the ratio in Daraw (48%) higher than in Shalateen (22%). Third, by conventional culture method, 20% of samples were positive to Staphylococcus spp. Fourth, Staphylococcal isolates were identified by conventional biochemical test, (46.7%) of these isolate showed positive for coagulase test. Fifth, these coagulase positive isolates submitted for molecular identification targeting 16S rRNA gene (Staphylococcus genus specific), nuc gene (S. aureus species specific) and Staphylococcal enterotoxins genes (SEA, SEB and SEC) by multiplex PCR, (92.9%) positive for 16s rRNA and confirmed as
Staphylococcus spp., (64.3%) positive for nuc gene and confirmed to be S.aureus and (50%) Staphylococcus isolates were enterotoxin-positive to sea, (35.7%) of them for S.aureus strains and (14.3%) for other coagulase positive Staphylococcus spp. Finally, these isolates were submitted for antibiotic sensitivity testing by using various antibiotics and the proportion of isolates resistance to the antibiotics were penicillin G and chloramphenicol (100%), colistin (93%), tetracycline and lincomycin (78.6%), oxalinic acid (71.4%), amoxicillin (50%), neomycin and cefaclor (42.8%), erythromycin (35.7%), ciprofloxacain (28.6%) and gentamycin (21.4%).

INTRODUCTION

Camel's milk can be considered as a good source of minerals, vitamins and characterized by higher ratio of lactoferrin than other dairy milk. Moreover, milk of camel could cover a big part of the daily needs of humans from these nutrients, because camel milk has most the essential nutrients (Al-Otaibi and El-Dermdash, 2013). In pastoral conditions, milk is always consumed fresh, in the raw state without heat treatments, and this consider as a health hazard to the consumer (Al-Majali et al., 2007).

Mastitis is a complex disease occurring worldwide among the dairy animals either in acute or in chronic form causing heavy economic losses, changes in the hygienic and compositional quality of milk and impairment of the technological properties of milk (Wielgosz- Groth and Groth, 2003) and decreased reproductive performance (Schrick et al., 2001). Additionally, mastitis can be harmful to suckling newborns. Acute and chronic mastitis can be clinically diagnosed by examination of the
udder, the milk, or by both (Obied et al., 1996). While detection of subclinical mastitis is difficult and depends on various indirect tests as California Mastitis Test and somatic cell count as well as microbial examination (IDF, 1987).

Bacterial infection are considered the primary cause of mastitis in domestic animals (Seifu and Bekele, 2010). S. aureus is one of the most important pathogen in milk or its products (LeLoir et al., 2003). S. aureus produces a wide variety of toxic proteins such as toxic shock syndrome toxin 1, exfoliative toxins, and enterotoxins (SEs). In addition to the five classical major antigenic types of SEs (SEA, SEB, SEC, SED, and SEE), four additional SEs (SEG, SEH, SEI, SEJ) have been also reported, and their corresponding genes have been described (Stephan et al., 2001; Kuzma et al., 2003). The staphylococcal enterotoxins are known as being agents of intoxication such as staphylococcal food poisoning in man and they may cause other types of infections (Gilmour and Harvey, 1990).

It is relevant to consider that the transmission of S. aureus is possible either by direct contact with animals or through contaminated food as milk or cheese (Vautor et al., 2005). Today 90% of human S. aureus are penicillin resistant (Zinn CE, 1999).

The current study aimed to evaluate percent of subclinical mastitis and risk factors associated with it in she-camel, Conventional bacteriological analysis of the mastitis pathogens, Detection of the presence of nuc gene of S. aureus and investigate the enterotoxins sea, seb and sec genes and application of antimicrobial sensitivity test to evaluate the rate of antibiotic resistance in different Staphylococcus aureus isolates.
MATERIALS AND METHODS

1.1. Sampling

A total of 150 raw milk samples were collected from 150 she camels from different localities, 100 from Daraw and 50 from Shalateen. These raw milk samples of she camels were collected during the period from June 2014 to January 2016. Collection, transportation and preparation of samples were based on the guideline described by (NMC, 1990).

1.2. California Mastitis Test (CMT) according to (Schalm and Noorlander, 1957).

1.3. Culturing method according to (Lancett and Bennett, 2001).

1.4. Coagulase test according to (Cookson, 1997).

1.5. Polymerase chain reaction (PCR).

a. DNA Extraction:

Total DNA extraction was carried out by a rapid boiling method according to (Reischl et al., 1994).

b. Multiplex PCR assay:

Multiplex PCR assay targeting 16S rRNA gene (Staphylococcus genus specific), nuc gene (S.aureus species specific), SEA, SEB and SEC was performed. The amplification was performed on thermal cycler by using total volume of 25ul reaction mix contain 5ul of template DNA, 20 pmol of each primer and 1X of PCR mix. Detailed sequences of primers and cycling protocols are depicted in (Tables 1,2). The analysis of PCR products was carried out using 1.5% ethidium bromide stained agarose gel.

c. Agarose gel electrophoresis was carried out according to (Sambrook and Russel, 2001).
Table (1): Primer Sequences used in PCR assay.

| Target       | Name (strand) | Primer sequence (5' - 3')                                      | Reference                  |
|--------------|---------------|----------------------------------------------------------------|---------------------------|
| Staphylococcus | 16S rRNA- F   | 5' - GGA GTG GGC AAG CGT TAT CC -3'                              | Monday and Bohach, (1999)  |
|              | 16S rRNA-R    | 5' - CGC ACA TCA GCG TCA G -3'                                   |                           |
| S. aureus    | Nuc -F        | 5' - GCG ATT GAT GGT GAT ACG GTT-3'                              | Brakstad et al., (1992)   |
|              | Nuc -R        | 5' - AGC CAA GCC TTG ACG AAC TAA AGC-3'                           |                           |
| SEA          | SEA-F         | 5'- TAAGGAGGTTGGTGCCCTATGG -3'                                   | Cremonesi et.al., (2005)  |
|              | SEA-R         | 5'- CATCGAAACCAGCcaaAGTT-3'                                      |                           |
| SEB          | SEB-F         | 5'- TCGCATCAAACGTGACAAACG-3'                                     | Johnson et al., (1991)    |
|              | SEB-R         | 5'- GCAGGTACTCTATAAGTGCC-3'                                      |                           |
| SEC          | SEC-F         | 5'- ACCAGACCCCTATGCAGATG-3'                                      | Cremonesi et.al., (2005)  |
|              | SEC-R         | 5'- TCCCATATCAAGGTGGTTCC-3'                                      |                           |

Table (2): Cycling protocols of PCR assay.

| Target     | Amplicon size | Cycling program                  |
|------------|---------------|----------------------------------|
| 16S rDNA gene | 228bp         | Step: Initial denaturation 94°C 4 min One cycle |
| Nuc gene   | 279bp         | Step: Denaturation 94°C 45s      |
| SEA        | 180bp         | Step: Anealing 55°C 45s          | 35 cycles               |
| SEB        | 478bp         | Step: Extention 72°C 45s         |
| SEC        | 371bp         | Step: Final extension 72°C 10min One cycles |

1.6. **Antimicrobial susceptibility test** was carried out according to the guidelines stipulated by *(NCCLS, 2001)*.
RESULTS

In this study, 59/150 (39.3%) of milk samples collected from she camels were detected with subclinical mastitis, 48/100 (48%) from Daraw, 11/50 (22%) from Shalateen, depicted in table (3). Table (4) showing the frequency of the isolated Staphylococci from the examined milk samples from subclinical mastitis, 30/150 (20%) Staphylococcus spp isolated from milk samples. Identification of these isolates was performed using phenotypic and genotypic methods. Coagulase test was conducted to 30 positive isolates resulting 14/30 (46.7%) coagulase positive Staphylococci CPS and 16/30 (53.3%) coagulase negative staphylococci CNS, depicted in table (4). The performed multiplex PCR assay for 14 coagulase positive isolates confirmed 13/14 (92.9%) of isolates to be Staphylococci by successful amplification of the 228 bp PCR product of the staphylococcal specific 16s rRNA gene. using the same multiplex PCR, 9/14 (64.3%) of isolates were confirmed to be S.aureus by successful amplification of 279 bp PCR product of the S.aureus specific nuc gene, 7/14 (50%) of isolates were confirmed to carry sea gene by successful amplification of 180 bp of PCR product of specific sea gene, 5/14 (35.7%) of isolates were confirmed to carry sea gene with S.aureus and 2/14 (14.2) of isolates were confirmed to carry sea gene with other staphylococci, photo (1) showing ethidium bromide stained 1.5% agarose gel electrophoresis of multiplex PCR assay. The antimicrobial sensitivity testing of isolated strains (n=14) to various antimicrobials revealed that (100%) of isolates were resistant to
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penicillin G and chloramphenicol followed by colistin (93%), tetracycline and lincomycin (78.6%), oxalinic acid (71.4%), amoxicillin (50%). The isolates show sensitivity to gentamycin (64.3%), neomycin and cefaclor (42.8%). The test show intermediate susceptible result to ciprofloxacin (64.3%) and erythromycin (50%), explained in table (5).

**Table (3):** prevalence of subclinical mastitis in she camels according to CMT

| Samples                  | NO. | Positive CMT | Score3 +ve | Score2 +ve | Score1 + ve | Negative CMT | Score 0 _ ve | Score trace _ ve |
|--------------------------|-----|--------------|------------|------------|-------------|---------------|--------------|----------------|
| Total no. of tested milk samples | 150 | 59           | 39         | 8          | 12          | 91            | 52           | 44             |
|                          |     | (39.3%)      | (26%)      | (5.3%)     | (8%)        | (60.7%)       | (34.7%)      | (29.3%)        |
| Daraw samples            | 100 | 48           | 33         | 8          | 7           | 52            | 28           | 24             |
|                          |     | (48%)        | (33%)      | (8%)       | (7%)        | (52%)         | (28%)        | (24%)          |
| Shalaten samples         | 50  | 11           | 6          | 0          | 5           | 39            | 24           | 15             |
|                          |     | (22%)        | (12%)      | (0%)       | (10%)       | (78%)         | (48%)        | (30%)          |

**Table (4):** frequency of the isolated *S.aureus* from the examined milk samples from subclinical mastitis

| Samples          | No. of tested milk samples | Positive Samples | Coagulase Positive *S.aureus* | Coagulase negative staph. | Negative samples |
|------------------|-----------------------------|-------------------|-------------------------------|---------------------------|------------------|
| Total no.        | 150                         | 30                | 14                            | 16                        | 120              |
|                  | (20%)                       |                   | (9.3%)                        | (10.7%)                   | (80%)            |
| Daraw samples    | 100                         | 19                | 8                             | 11                        | 81               |
|                  | (19%)                       |                   | (8%)                          | (11%)                     | (81%)            |
| Shalateen samples| 50                          | 11                | 6                             | 5                         | 39               |
|                  | (22%)                       |                   | (12%)                         | (10%)                     | (78%)            |
**photo (1):** Ethidium bromide stained 1.5% agarose gel electrophoresis of multiplex PCR assay. Lane M:100bp DNA ladder, Lane 1: Positive control contain 3 band (180bp of Sea gene, 228bp of 16S rRNA gene and 279bp of Nuc gene), Lanes 2,3,11 and 14: *Staphylococcal aureus* not contain Sea gene Samples, Lanes 4 and 5: Staphylococcal but not *aureus* samples, Lanes 6,8,9,10 and 13: *Staphylococcal aureus* contain Sea gene Samples, Lanes 7 and 12: Staphylococcal but not *aureus* contain Sea gene Samples, Lane 15: Negative samples, Lane 16: Negative control.

**Table (5):** Shown Percentage of sensitive, intermediate and resist samples to different antibiotics.

| Antibiotics          | NO. of sensitive samples | NO. of intermediate samples | NO. of Resist samples |
|----------------------|--------------------------|------------------------------|-----------------------|
| Penicillin G         | 0 (0%)                   | 0 (0%)                      | 14 (100%)            |
| Neomycin N           | 6 (42.8%)                | 2 (14.3%)                   | 6 (42.8%)            |
| Chloramphinicol C    | 0 (0%)                   | 0 (0%)                      | 14 (100%)            |
| Colistin CT          | 0 (0%)                   | 1 (7.1%)                    | 13 (93%)             |
| Amoxicillin AX       | 2 (14.3%)                | 5 (35.7%)                   | 7 (50%)              |
| Gentamycin CN        | 9 (64.3%)                | 2 (14.3%)                   | 3 (21.4%)            |
| Erythromycin E       | 2 (14.3%)                | 7 (50%)                     | 5 (35.7%)            |
| Oxalinic acid OA     | 4 (28.6%)                | 0 (0%)                      | 10 (71.4%)           |
| Cefator CEC          | 6 (42.8%)                | 2 (14.3%)                   | 6 (42.8%)            |
| Tetracycline TE      | 0 (0%)                   | 3 (21.3%)                   | 11 (78.6%)           |
| Lincomycin L         | 2 (14.3%)                | 1 (7.1%)                    | 11 (78.6%)           |
| Ciprafloxacin CIP    | 1 (7.1%)                 | 9 (64.3%)                   | 4 (28.6%)            |
DISCUSSION

In present study, the clinical examination of 150 she camels, 100 from Daraw and 50 from Shalateen revealed that all examined animals have no clinical signs related to mastitis.

According to (Shearer and Herris, 2003) subclinical mastitis is important due to the fact that it is 15 to 40 times more prevalent than clinical form, it usually precedes the clinical form, it is difficult to detect and adversely affects milk quality and production, beside that it consider a reservoir of microorganisms that lead to infection of other animals within the herds. Losses due to mastitis may even be high in developed countries because mastitis prevention practices like post milking dipping of teat and dry period therapy are not so far being carried out. California Mastitis Test can be used as screening test to detect sub-clinically infected udders of female camels (Bekele and Molla, 2001), as the degree of gel formation is related with the number of cells in milk (Abdurahman, 2006). CMT scores may varied based on severity of inflammation. The percent of sublinical cases of mastitis in Daraw (48%) was higher than that in Shalateen (22%). This variation may be attributed to the difference of geographical area and individual herd management (Guidry, 1985).

In present study the data of CMT showed that, the infection of udder in she camels was mainly subclinical and the prevalence of infection in Daraw and Shalateen was higher than that recorded by (Saber et al., 2010) who concluded that, the prevalence of subclinical mastitis was (9.52%), such variation may be attributed to the environmental factors and management that play a significant role in the
prevalence of subclinical mastitis (Abdurahman, 1996). Furthermore, in Daraw, camels were housed in closed places with other animals that may help in transmission of infection from other dairy animals. In addition to poor management lead to the high prevalence of the disease in the camel herds. In Shalateen the dry weather and rearing animals in open area may be the cause of decreasing the prevalence of infection in this area. However, the existence of the ticks and thorny plants may cause injury to the udder causing tissue damage that facilitate the entrance of microorganisms into udder (Woubit et al., 2001). These results revealed that, it is cheaper and easier to prevent mastitis by improving hygienic measures and culling chronically infected camels to eliminate important pathogen reservoirs, than to treat by medication and increase the cost of treatment including veterinary fees, medicines, risk of quackery and loss of milk production. In addition to that, the treatment also contribute to the buildup of antibiotic resistance.

The PCR method compared to conventional methods of identification of S.aureus isolates, the PCR method is less laborious and more accurate. In the future it is likely to be the predominant method of identification of pathogenic bacteria. In the present study, the identification of S.aureus isolates was performed conventionally and with the PCR method and detection of staphylococcal enterotoxins (sea, seb, sec) was performed using PCR method.

The current study indicated that, using culture method identified about 20% of examined samples infected with staphylococcal isolates including (9.3%) S.aureus. These results were close to some extent to that, recorded by (Abdel Hameid et al., 2004), who recorded that 14% of
their tested samples were positive to *S. aureus*, while the results of this study were lower than the results of *(Türkyilmaz et al., 2010)* and *(El-jakee et al., 2008)* who recorded 22.9% and 24.8%, respectively were positive to *S. aureus*.

Identification of these isolates was performed using biochemical method, 46.7% of these isolate showed positive for coagulase test. These results were higher than that previously reported by *(Abdel All et al., 2010)* as he recorded that 33.6% of isolates were positive for coagulase test. the present results were lower than *(Mohamed, 2012)* as he recorded 98.5% of isolates were positive for coagulase test.

All our isolates were not confirmed as *S. aureus* based on results from PCR, this may indicate that, PCR assay is more accurate test for detection of coagulation of *S. aureus* rather than, coagulase test, however, the later test is cheaper than the former technique. The production of coagulases is not unique feature of *S. aureus* but are shared by *S. intermedius* and *S. hyicus* *(El-Jakee et al., 2008)*.

The results of PCR in this study revealed that, the positive samples for *Staphylococcus* spp were 13 (92.9%) out of 14 isolates. Subclinical staphylococcal mastitis obtained in this study as a result of PCR (8.66%), this result is coincides to *(Sindhu et al., 2010)* who stated that (6.58%) of milk samples contain *Staphylococcus* spp by PCR. *S. aureus* are 9 (64.3%) out of 14 isolates. subclinical mastitis caused by *S. aureus* (6%), this result similar to *(Yesim and Haluk, 2012)* was (6%) and close to results of *(Fox and Gay, 1993)* as they stated that *S. aureus* mastitis ranged from (7-40%), while it was lower than that, recorded by *(Janosi and Balty, 2004)* who recorded 80%.
The presence of 3 enterotoxin genes (sea, seb and sec) was tested in 14 coagulase-positive Staphylococcus (CPS). 7 (50%) Staphylococcus isolates were enterotoxin-positive to sea, 5 (35.7%) of them for S.aureus strains and 2 (14.3%) for other coagulase positive spp. and these result close to (Da Cunha et al., 2007) as they stated that amongst the predominant Staphylococcus species, 100 % of the S. simulans, 64.7 % of S. epidermidis, 38.5 % of S. saprophyticus and 22.7 % of S. aureus harboured enterotoxin genes. S. epidermidis, S. simulans, S. saprophyticus, S. hyicus and S. lentus have been reported elsewhere to contain enterotoxigenic genes.

In the present study staphylococcal enterotoxin (SEA) 50% of subclinical mastitis. This result was similar to (Balaban and Rasooly, 2000) who recorded that the sea gene was the most prevalent gene. SEA is considered to be a primary cause of food poisoning (Cha et al., 2006).

This study concluded that raw milk may contain very dangerous pathogenic bacteria that make milk unsafe, capable of causing milk borne diseases.

The antimicrobial sensitivity test is important guide to the veterinarian in selecting the most appropriate antimicrobial agent for treatment of clinical mastitis and subclinical mastitis caused by S.aureus. The antimicrobial sensitivity testing of isolated strains (n=14) to various antimicrobials revealed that,(100%) of isolates were resistant to penicillin G and chloramphenicol followed by colistin (93%), tetracycline and lincomycin (78.6%), oxalinic acid (71.4%), amoxicillin (50%). The isolates showed that, the susceptibility to gentamycin (64.3%), neomycin and cefaclor (42.8%). The test show intermediate
susceptible result to ciprofloxacin (64.3%) and erythromycin (50%). The results of penicillin (100%) were close to result of (Abera et al., 2010) (94.4%), but higher than (Ebrahimi et al., 2009) (78.5%) and (Moroni et al., 2006) (69.1%). The result of erythromycin (35.7%) were closer to the result obtained from (Adwan, 2006) (40.9%), but higher than those reported by (Mohamed, 2012) (15.7%) and (Saad et al., 2007) (13.8%). The result of amoxicillin (50%) is higher than those reported by (Mohamed, 2012) (34.2%), but less than the results obtained from (Moroni et al., 2006) (100%) and (Klimien et al., 2011) (81.3%). In this study the antimicrobial susceptibility test of the staphylococcal isolates indicated that, most of isolates were susceptible to antimicrobial agents such as gentamycin, cephaclor followed by ciprofloxacin which are a new drugs. The susceptibility of isolates to antibacterial varies from strain to strain and from region to another, therefore the obtained data are in agreement with the results of Iraqi study (Al-Ani and Al-Shareefi, 1997). The ideal properties of drug selected for treatment of mastitis must has good tissue penetration, a low degree of protein binding, low irritancy and a short milk with holding time (Mody et al., 1998).

In conclusion S.aureus subclinical mastitis was common in milking she camels in Daraw and Shalateen affecting milk yield and consumer safety. California Mastitis Test, culture method and biochemical tests were not enough for detection of S.aureus subclinical mastitis. PCR assay is accurate, rapid and specific method for detection of S.aureus subclinical mastitis in milking she-camels. The caution and restricted prescription is recommended in order to avoid development of resistant bacterial strains and to avoid antibiotic residues in milk.
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