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A PRELIMINARY SURVEY OF *STAPHYLOCOCCUS AUREUS* INFECTION IN GOATS WITH MASTITIS IN "*NOUQRA*" VALLEY OF ASWAN GOVERNORATE, SOUTH EGYPT

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This article is an abstracted outline from MV-theses presented by the fourth author to Faculty of Vet. Med., Dept. Animal Med., (Infectious Diseases), South Valley University under supervision of the firs-three authors

Received: 30 December 2018; **Accepted:** 23 January 2019

ABSTRACT

Prevalence of mastitis in goats located in *Nouqra* valley, Aswan Governorate, by indirect method (California Mastitis Test) in paralleling with culturing and molecular (PCR) procedures for detection of *Staphylococcus aureus* as a major mastitis pathogen. A total of 148 raw milk samples were subsequently collected from apparently healthy native breed goats, with different age and parity, and subjected to California Mastitis Test. By CMT, our results indicated that 117 (79.05 %) of the tested—samples were positive. Culturally using Baired Parker media, pure strains of *Staphylococcus aureus* was isolated from CMT—positive samples with a percentage of 1.35% and 77.7% of the samples showed fair growth, which classified as other *non-aureus staphylococci.* Coagulase test revealed 7 (4.7%) samples were positive and 141 (95.3%) were negative. These isolates were molecularly tested using 16s Rrna (*Staphylococcus* genus specific), nuc gene (*S.aureus* species specific) and mecA gene (methicillin resistance gene) by Multiplex PCR Technique. Their results indicated that 87.5% were positive for *16s Rrna*, 25% were positive for *nuc* gene, 75% were positive for *mec A* gene and 12.5% were negative. The in-vitro antibiotic sensitivity testing revealed that the resistant percentages to penicillin were surprise (100% resistance). Amoxicillin, cefaclore, colistin, oxolinic acid, neomycin, erythromycin, and lincomycin were also examined with various resistant results. Approx. 85 % (85.71%) of the tested strains were Ciprofloxacin—sensitive.

***Key words**: Preliminary survey, Staph aureus, antibiotic resistance, mecA, Nuc genes, mastitis*

INTRODUCTION

Mastitis is still frequently incriminated as one of the most important threats affecting the world's dairy industry (Serrano-Rodríguez, 2017) inducing colossal damages to livestock production (Samiullah *et al.*, 2000). There are two form of mastitis; clinical and subclinical forms. The later appears to be more prominent (Willium *et al.*, 1987). Various pathogens are encountered as etiologic agent of mastitis. Based on the principal reservoirs of mastitogens, mastitis was into environmental and contagious mastitis (Hogan and Smith, 2012). The later appears to be more prominent than the former. *Staphylococcus aureus* is frequently incriminated as a serious mastitogen of milk producing goats and ewes, particularly in sublevel hygienic measures (Salaberry SR, 2015 and Serrano-Rodríguez, 2017).

Small ruminants particularly goats are more populated animals than other ruminants in El Nouqra valley. This valley is one of the oldest Egyptian valleys located in Eastern border of Nasr El Nouba and Draw centers, neighbor to "Khirt Valley" of Aswan Governorate and west to the desert of the Red Sea Governorate in South Egypt (GIS, 2013), (Fig. 1).

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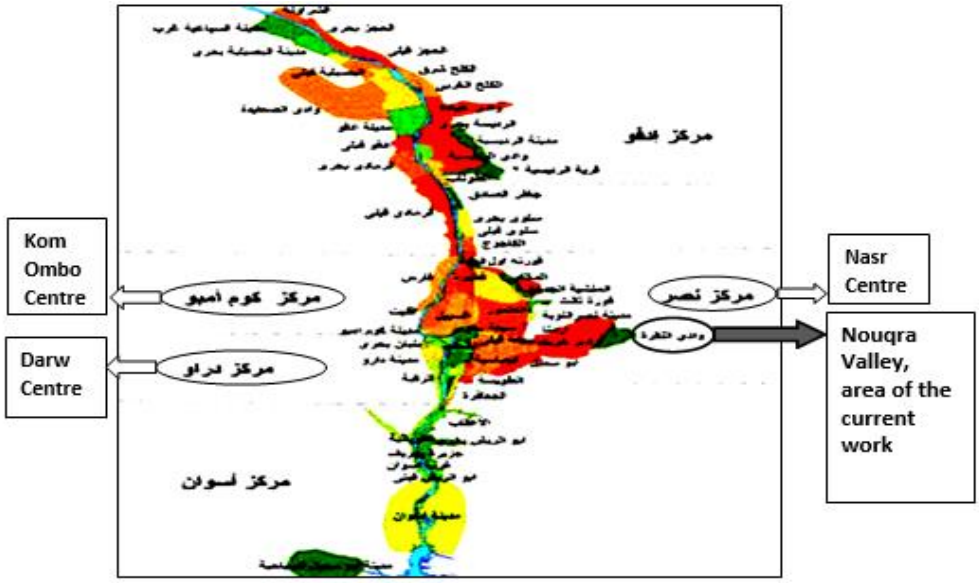

Fig. 1: Centers of Aswan Governorate indicating the location of "Nouqra" Valley

The average public economic income of the working people in Nouqra valley is low (GIS, 2013) and the most people prefers goats for milk and meat productions. Moreover, Nouqra's peoples assumed that goats have more resistance to dry and harsh environmental conditions in comparison with sheep and other large ruminates. In Nouqra valley, goat's milk considers a preeminent food with a considerable level of nutritional value. Therefore, the aim of the current work was carried out to reveal-up the prevalence of subclinical mastitis in goats by indirect method (California Mastitis Test) in association with culturing technique focusing on *Staphylococcus aureus*. The isolated strains were molecularly tested to *mecA gene and nuc gene* of *Staphylococcus aureus* using PCR with species specific primers. In-vitro antibiotic sensitivity tests for the isolated Staphylococcus aureus was also done.

MATERIALS AND METHODS

A total of 148 milk samples were subsequently collected from goats of local breeds apparent normal goats with different age and parity Table (1& 3), There were four cases (2.7%) with clinical mastitis and 144 (97.3%) were apparent healthy cases. Milk samples were collected in sterile single use disposable falcon tubes with tightly fitted caps, all samples subjected to California Mastitis Test, then frozen immediately at -20^{o}c (Pamela, 2005).

Table 1: Age-wise distribution of goat age and their percentage.

Age (years)	No. of female goats	% to all (n =148)
>1 – 2	58	39.19
>2 – 3	41	27.70
>3 – 4	18	12.16
> 4	31	20.94
Total	148	100

Table 2: Average number of birth (parity).

Parity	No. of female goats	% to all (n =148)
1 birth	23	15.54
2 birth	41	27.70
3 birth	32	21.62
4 birth	30	20.27
5 birth	22	14.86
Total	148	100

Culturing of S.aureus

S.aureus was confirmed on baird-parker media according to (Lancette and Bannette, 2001), typical large black colonies appeared and 3-4 colonies kept in glycerol broth at -70 to-80^{o}c for further identification. The coagulase test was performed by two different methods; the slide and tube coagulase test (Cookson, 1997).

Antibiotic sensitivity test

S.aureus strains which had been isolated and confirmed with coagulase test (positive samples) had been tested for it's susceptibility to antibiotics by disc diffusion method (Bauer *et al.*, 1966) and (Dereesse *et al.*, 2012), by using Muller Hinton Agar and the diameter of the zones were measured and compared (NCCLS, 2001).

Primer used in PCR assay:

Application of PCR for identification of 16srRNA, nuc gene (general primer) and mec A gene of *S. aureus* was carried out by using Primers as showen in the following table:

Table 3: Primers used in PCR assays.

Target	Name (strand)	Primer sequence (5 - 3)	Reference
Staphylococcus	16S rRNA -F	5'-GTA GGT GGC AAG CGTTAT CC -3'	Monday and Bohach (1999)
	16S rRNA -R	5'- CGC ACA TCA GCG TCA G -3'	
Staph aureus	Nuc 1	5'- GCG ATT GAT GGT GAT ACG GTT-3'	Brakstad *et al.* (1992)
	Nuc 2	5'- AGC CAA GCC TTG ACG AAC TAA AGC-3'	
Methicillin resistance	Mec A- F	5'-GTG AAG ATA TAC CAA GTG ATT-3'	Zhang *et al.* (2005)
	Mec A- R	5'-ATG CGC TAT AGA TTG AAA GGA T-3'	

Detection of *16s rRNA, mecA* gene *and nuc* gene

Using Multiplex PCR method for detection of 16s rRNA of *Staphylococcus* genus specific, nuc gene for *S.aureus* species specific and mecA gene for detection of methicillin resistant *S.aureus.*

1. DNA extraction: A boiling procedure to the pellet at 100^{0}c for 20 minutes was used to extract DNA from bacterial isolates according to Reischl *et al.* (1994).

2. DNA amplification reaction: Multiplex PCR assay was performed By using total volume of 25ul reaction mix contain 5ul of template DNA, 20 pmol of each primer and 1X of PCR mix. The PCR cycles were carried out in Eppendorf AG (22331 Hamburg) thermocycler. The analysis of PCR products was carried out using 1.5% ethidium bromide stained agarose gel. This technique consists of repetitive cycles, where each cycle of PCR synthesis involves three steps: heat denaturation, annealing and extension.

3. Agarose gel electrophoresis: The agarose gel electrophoresis was performed according to Sambrook and Russell (2001). **First:** agarose gel is prepared and casted with concentration appropriate for the size of DNA fragments to be separated. **Second**: the DNA samples are loaded into the sample wells and the gel is run at a voltage and for a time period that will achieve optimal separation. **Third:** the gel is stained either by incorporation of ethidium bromide into the gel or electrophoresis buffer during electrophoresis or by submerging in buffer containing ethidium bromide after electrophoresis, then visualized directly upon illumination with UV light.

RESULT

All samples collected were tested by California Mastitis Test (CMT) as shown in table (4).

Table 4: CMT of collected milk samples.

Samples	CMT positive		CMT negative	
148	NO.	%	NO.	%
	117	79.1	31	20.9

By conventional culture methods on baired parker agar (table5), Two (2) samples show the characteristic colonial growth of *S.aureus*, 115 samples show fair growth, small black colonies and no clear zones (other *staphylococci)*. Thirty one samples were negative.

Table 5: Frequency of the isolated *S. aureus* from the examined milk samples.

NO. of tested milk samples	Positive				Negative	
	S. aureus		Other *staphylococci*			
148	NO.	%	NO.	%	NO.	%
	2	1.35	115	77.7	31	20.9

All *staphylococci* isolates were tested by slide and tube coagulase test to differentiate between coagulase positive and coagulase negative staphylococci, and found that 7 samples are positive for coagulase test and 110 samples negative for coagulase test as shown in table (6). Coagulase positive staphylococci isolates were tested for sensitivity test to 12 different antimicrobials as shown in Table (7).

All *Staphylococcus* spp. isolated from examined raw milk samples were subjected to PCR (Multiplex PCR) for detection of 16s rRna (for *Staphylococcus* genus specific), nuc gene (*S.aureus* species specific) and mecA gene (methicillin resistance gene), two isolates of total 7 isolates tested confirmed as *S. aureus* (table 8). Six (6) isolates are positive for mecA gene. Which appear as clear bands on agarose gel at a74bp compared to molecular weight marker (Figures 1, 2).

Table 6: Results of coagulase test.

N0. of sample	Coagulase(+ve)		Coagulase(-ve)	
	NO.	%	NO.	%
117	7	6	110	94

Table 7: The frequency of resistance to various antimicrobials (n=7 isolates).

Antimicrobial agents	S		I		R	
	NO.	%	NO.	%	NO	%
Amoxicillin (AX)	1	14.29	-	-	6	85.71
Cefaclore (CEC)	1	14.29	-	-	6	85.71
Ciprofloxacin (CIP)	4	57.14	2	85.57	1	14.29
Colistin (CT)	1	14.29	-	-	6	85.71
Erythromycin (E)	5	71.43	1	14.29	1	14.29
Lincomycin (L)	5	71.43	1	14.29	1	14.29
Neomycin (N)	2	28.57	1	14.29	4	57.14
Oxolinic acid (OA)	1	14.29	-	-	6	85.71
Penicillin (P)	-	-	-	-	7	100
Tetracyclin (TE)	1	14.29	-	-	6	85.71

S. Susceptible I. Intermediate R. Resistant

Table 8: *S. aureus* isolates as diagnosed by PCR method.

No. of the tested *S.aureus*	Positive		Negative	
	NO.	%	NO.	%
8	2	25	6	75

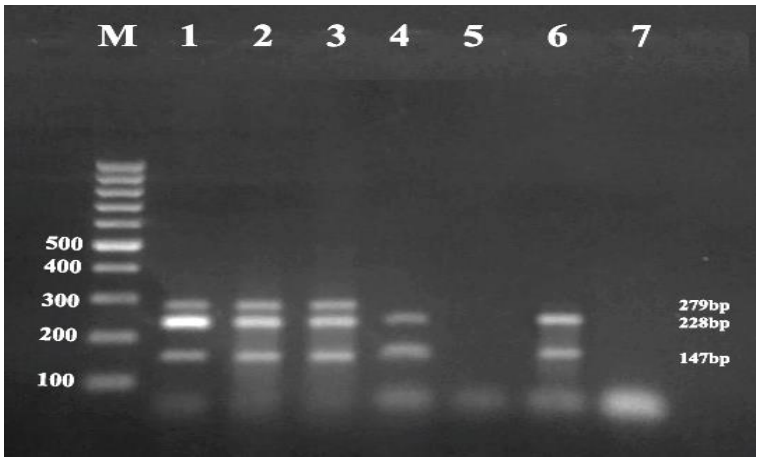

Figure (1): PCR of nuc and mecA gene on agarosegele electrophoresis. **Lane M:** 100bp DNA plus ladder, **Lane 1:** Positive control contain 3 band (147, 228 and 279bp), **Lane 2, 3:** isolates contain 3 bands of (147, 228 and 279bp) of staphylococcus aureus and mec A gene, **Lane 4, 6:** contain two band of (228 and 147bp) of staphylococcus but not auerus and mec A gene, **Lane 5:** Negative sample, **Lane 7:** Negative control.

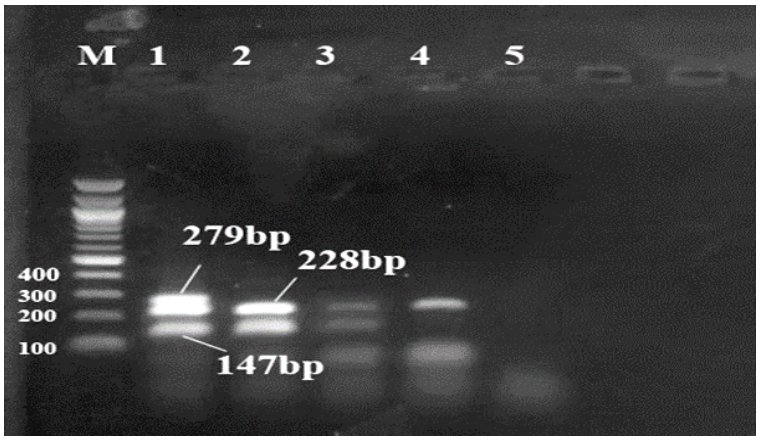

Figure (2): PCR of nuc and mecA gene on agarose gel electrophoresis. **Lane M:** 100bp DNA plus ladder, **Lane 1:** Positive control contains 3 band (147, 228 and 279), **Lane 2, 3:** isolates contain bands of (228 and 147) of staphylococcus but not auerus and ***mec A gene***, **Lane 4:** contain one band of 228 of staphylococcus but not auerus, **Lane 5:** Negative control.

DISCUSSION

Staphylococcus aureus—Mastitis is a widespread disease of milk producing animals including goats (Salaberry, 2015) and is associated with a significant reduction in milk yield and deteriorated milk quality. The disease results in partial or complete damage to udder tissues and decreases the productive life span of the animal (Gonzalez *et al.,* 1980).

Currently, culturing 148 milk samples collected from mastitic and apparent normal udder of dairy goats indicated that 117(79.1%) samples gave positive result with California mastitiic test (CMT) and appear growth on baird parker agar media. Other microorganisms can produce black colonies on baired parker agar media as Enterococcus fecalis and proteus mirabilis (Baired-parker, 1992). This is agreement with (Maya *et al.,* 2013) who found other staphylococci grow on baired parker agar as (S. schleiferi). and dis agreement with (Al-azem *et al.,* 2013) who recorded a higher percentage of isolated strains were *S. aureus* (95.5%) on baired parker agar.7 isolates are staphylococcus species which gave positive result with coagulase test. The production of coagulases and thermonucleases are not unique features of S.aureus but are shared by S.intermedius and S.hyicus (El- Jakee *et al.,* 2008).

Prevalence of subclinical mastitis of goat's raw milk obtained from Nokra Valley, Aswan Governorate, Egypt is 76.35%this result is similar to that observed by (Vasiu, 2008) as he recorded the prevalence of subclinical mastitis was 70.21%, and higher than that recorded by (Contreras *et al.,* 2007; Leitner *et al.,* 2008; Bagnika *et al.,* 2011). They found the prevalence of subclinical mastitis in goat is usually between 5 to 30%. Indiscriminate use of antibiotics has led to ineffectiveness of antibiotic treatment (Ali *et al.,* 2010).

Resistance of staphylococci to methicillin and all β-lactam antibiotics is associated with the low affinity of a penicillin-binding protein, PBP2a, which is not

present in susceptible staphylococci. Pierre *et al.* (1990); Unal *et al.,* 1992; Chamber, (1997). This protein is encoded by the mecA gene. Mastsuhashi (1986), is the corner-stone responsible for producing MRSA phenomenon (Ubkata *et al.,* 1989; Berger-Bachi 1997).

It is concluded that, *Staphylococcus aureus*—subclinical mastitis is seriousness problem in goat's population in the area of study. The misuse of antimicrobial agents leading to the development of resistant isolates which may be transmitted to the human beings causing somber troubles. Amplification of DNA by PCR is a rapid and sensitive method for the detection of specific DNA sequences**.**

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مسح مبدئي لعدوى المكور العنقودي الذهبي في الماعز المصاب بالتهاب الضرع بوادي النقرة ـ بمحافظة أسوان ، جنوب مصر

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الغرض من البحث معرفة معدل أنتشار الميكروب العنقودي الذهبي المسبب للألتهاب الضرع الخفي والظاهري في الماعز بمنطقة وادي النقرة المجاور لوادي خريت بمحافظ أسوان بجنوب مصر. أجري البحث على عدد ١٤٨ حالة من الماعز متعدد الأعمار والتي أختبرت بأختبار كاليفورنيا اللبن واتضح ان عدد ١١٧ بنسبة ٧٩,٠٥ % كانت إيجابية وان ميكروب العنقودي الذهبي كان سائدا. وأن العترات المعزولة تم اختبارها PCR المتعدد لمعرفة بعض الجينات امسءولة عن تصنيف و ضراوة الميكروب العنقودي الذهبي واتضح ان 87.5% من العترات المختبرة كانت إيجابية لـ (*16s Rrna*) وأن ٢٥ % منها كانت أيجابية لـ (*nuc gene*). ومن ناحية أخرى أوضحت الأختبارات ان معظم العترات المختبرة (٧٥ %) كانت تحتوي على جين (*mec A* gene) المقاوم لمجموعة البنسيلين. هذا وقد نوقشت نتائج وجود هذه الجينات بالعترات المعزولة. وقد أوجزت نتائج أختبار الحساسية لبعض العترات المعزولة انها شديدة الحساسية لمركب سيبروفلوكساسين ومقاومة بدرجات متفاوتة للعديد من المضادات الحيوية التي تستخدم في علاج التهاب الضرع خاصة البنسيلين والأمكساسللين والسيفاكلور والايرثرميسين وغيرهم.