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Prevalence of Subclinical Mastitis in Small Ruminants and Role of Staphylococcus Species in Such Infection

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ABSTRACT

The current study was carried out on a number of sheep and goats farms during the period extended from January 2016 till the end of December 2017 in order to estimate the prevalence of subclinical mastitis in small ruminants and highlight the role of *Staphylococcus* species in inducing such infection. For this aim, about 940 milk samples collected from sheep and goats' farms (455 samples/ sheep and 485 samples/ goats) were subjected to California Mastitis test (CMT) to detect the prevalence of subclinical mastitis, then positive CMT samples in addition to; 100 bedding samples (50 samples / each species) collected from different sites of animal's yards particularly from wetted areas with high moisture and organic matter load and 100 water samples collected from different sites of animal's yards (50 samples / each species) were subjected to the ordinary bacterial culturing steps for isolation and identification of Staphylococcus species. The results revealed prevalence of subclinical mastitis estimated by 32.96% and 30.92% in sheep and Goat farms, respectively. The total prevalence of Staphylococci in Sheep farms was 27.2% where *S. aureus* accounted for 12.4% and Coagulase Negative Staphylococci (CNS) represented the highest percentage (14.8%). The highest isolation rate recorded in bedding samples (34.0%) followed by milk samples (26.0%) and water samples (24.0%), while in goats farms the prevalence of *Staphylococci* species was 32.0% (16.8% *S. aureus* and 15.2% CNS) with highest rate of isolation from bedding samples (36.0%) followed by milk samples (34.0%) then water samples (22.0%). In addition, the isolated Staphylococcus strains where tested for their sensitivity to certain commercial antibiotics using disc diffusion method. The results revealed complete resistant of isolated *S. aureus* to Enrofloxacin (5 µg) and Oxytetracycline (30 µg) and moderate sensitivity to Amoxicillin (10 µg) and Erythromycin (15 µg). While CNS showed complete resistant to Flumequine (30 µg), Doxycycline (30 µg) and Sulpha.-Trimethoprim (25 µg), in contrary, they were highly sensitive to Amoxicillin (10 µg) and Erythromycin (15 µg) antibiotics. At last, molecular detection of *clfA* gene specific for coagulase positive Staphylococci were performed.

1. INTRODUCTION:

Despite the low fame of milk and milk products that produced by small ruminants, they still one of the most important sources of protein and calcium of animal origin in many areas around the world specially developing countries and those of hard geographic nature that not suitable for cattle farming; as small ruminants have the ability to adapt with various climatic and environmental conditions.

The annual milk production from sheep and goats was estimated by 18 million tonnes worldwide (Haenlein, 2002). In Egypt and according to a statistics of FAO (1998), the population of sheep and goats has increased from 1989 to 1999 by 29.9% and 32.8%, respectively, that helped in increasing the milk production from sheep by 75% but no difference in goat's milk was recorded. Even though, this increase still unable to meet the demands of the growing population in Egypt which has a per capita protein and calcium of animal origin of 12 g and 141 mg, respectively (FAO, 1998) that represents a small proportion of the daily recommended consumption rates for normal person which are 60 g and 1000 mg, respectively (Haenlein and Abdellatif, 2004). So, the continuous developing of the livestock sector is inevitable and indispensable matter that occupies many minds in the Egyptian country for bridging the big gap between production and consumption rates in addition to participate in strengthening economy of this great country.

Unfortunately, many diseases hinder the development of animal production sector all over the world and specially in Egypt due to spreading of rural animal farming that lack biosecurity measures and the suitable veterinary management. Among those diseases, mastitis ranks as the most prevalent one between dairy animals, that is defined as inflammation of animal mammary gland due to infection with one or more of pathogenic agents. In case of small ruminants, the bacterial infection is the usual cause of that disease specially the infection with Staphylococcus species that is considered the most isolated bacteria from different types of small ruminant mastitis (Bergonier et al., 2003).

Infection with *S. aureus* is usually associated with clinical mastitis; however, the incidence of clinical mastitis is generally below 5% annually (Bergonier and Berthelot, 2003), this percentage may exceed the limit of 30-50% in sporadic outbreaks where *S. aureus* is one of the main accused, what result in mortality or culling of 70-90% of animals from the herd (Bergonier et al., 2003; Olechnowicz and Jaśkowski, 2014), moreover, acute infection with *S. aureus* results in developing cases of gangrenous mastitis specially in goats that lead to 30-40% mortalities of affected animals if not treated rapidly (Menzies and Ramanoon, 2001; Contreras et al., 2003). In addition to losses in animals, the infection with *S. aureus* has a public health concern due to its zoonotic importance; it produces heat stable enterotoxins in food that lead to poisoning of consumers (Le Loir et al., 2003).

On the other hand, subclinical mastitis is the most prevalent type between small ruminant and has incidence of 5-30% and according to other authors 9-50% (Contreras et al., 2007; Olechnowicz and Jaśkowski, 2014), however this type not cause animal deaths but its economic losses exceed that of clinical mastitis (Quinn et al., 2002). CNS is considered the main causative agent of this type by prevalence of 78% in dairy ewes and 71% in goats while *S. aureus* prevalence is 4% and 8%, respectively (Bergonier et al., 2003).

Udders, teats and surrounding skin are considered the main sources of CNS (Olechnowicz and Jaśkowski, 2014) where the infection develops through invasion the teat canal. With the presence of some factors such as having deep pendulous udder with high implanted teats, the infection probability may increase (Casu et al., 2010). Also, incorrect milking machines procedures give a chance to these opportunistic organisms to set the infection (Contreras et al., 2003). Usage of contaminated milking machines and equipment or even contaminated workers hands if hand milking is applied, in addition to suckling lambs and kids help in spreading of infection between herd animals.

Mastitis can be diagnosed by several ways but bacterial culture is considered the standard diagnostic method in small ruminants (Contreras et al., 2007), California Mastitis Test (CMT) also can be used to give good prediction about the subclinical infection existence in the dairy herd that confirmed later by bacterial culturing. In addition, somatic cell count (SCC) in milk is a convenient method of diagnosis in ewes where the animal is considered infected by subclinical mastitis if its milk contain 500.000 cell/ml or more (Kiossis et al., 2007), while this method not reliable in goats except with specific calibration as their milk content of somatic cells may induced by non-infectious causes such as estrus, season of milking, milk yield and stage of lactation (Olechnowicz and Jaśkowski, 2014).

Control of mastitis in the herd depends on three main axes; prober milking machine management and sanitation, culling of clinical, subclinical and chronically infected animals, finally application of antibiotic therapy during dry off period. Also, the role of pen hygiene management shouldn't be neglected as it helps in controlling the Staphylococcus infection even if indirectly (Bergonier et al., 2003). Application

of post-milking teat dipping is not a common procedure in small ruminants like in cattle; it is used mainly in case of infection outbreaks to reduce the infection spreading. So, it is clear that the main dependence in controlling that disease relays on usage of antibiotics that should be applied rationally to avoid the development of bacterial resistance.

This study aims to estimate the prevalence of subclinical mastitis cases in sheep and goat farms and evaluate the role of Staphylococcus species in inducing such infection, in addition to detect the antibiotic sensitivity to the isolated strains.

2. MATERIALS AND METHODS:

2.1.Collection and preparation of sample

2.1.1. Milk samples:

A total of 940 milk samples were collected from sheep and goats' farms (455 samples/ sheep and 485 samples/ goats) and subjected to California Mastitis test (CMT). After cleaning of udders, each teat end was scrubbed with a separate pledged of cotton moistened with betadine and the first few streams of milk were discarded. The positive CMT samples were placed in an ice box and transported directly to the laboratory. In a sterile test tube, 20 ml of each milk sample were centrifuged at 10.000 rpm for one minute to get the sediments that was added to 5 ml of nutrient broth at last. Samples were incubated at 37°C for 24 hours for microbiological examination (Schalm et al., 1971).

2.1.2. Bedding samples:

A total of 100 bedding samples were collected from different sites of animal's yards (50 samples from sheep yards and 50 samples from goats' yards) particularly from wetted areas with high moisture and organic matter load. Samples were taken at depth of 5 cm from soil surface in sterile glass bottle fitted with sterile glass stopper according to Clegg et al., (1983). Samples were transferred to the laboratory where each sample was subjected to thorough mixing, and then one gram was weighed and triturated well in a sterile mortar with 99 ml of sterile buffered peptone water (BPW) then aseptically strained through sterile gauze. The obtained filtrate was incubated at 37°C for 24 hours for microbiological examination.

2.1.3. Water samples (Moubarak, 1989):

100 water samples were collected from different sites of animal's yards (50 samples / each species) where 10 representative samples were taken from water in front of the animals per visit. Samples were collected by using sterile plastic syringes (20 ml capacity). Each sample was labeled and identified to its source, site and type of watering system.

2.2.Detection of subclinical mastitis using CMT:

Small amount of milk from each quarter of examined animals were subjected to is CMT according to Quinn et al., (2004) for detection of animals infected with subclinical cases.

2.3.Isolation and identification of *Staphylococcus aureus*:

Samples were incubated in nutrient broth at 37°C for 18-24 hours, and then loopful from each incubated sample was streaked directly onto Mannitol salt agar plates. The plates were incubated at 37°C for 24-48 hours under aerobic conditions (Cruickshank et al., 1975). The suspected colonies that grow on incubated plates were picked up and subculture onto nutrient agar slants and incubated at 37°C for 24 hours then stored in refrigerator for more identification. The isolated colonies were subjected to staining with Gram's stain for detection the clusters of Gram positive cocci. Moreover, biochemical characteristics of purified *Staphylococcus* colonies were identified by using catalase test, tube coagulase test, oxidation fermentation test; finally, hemolytic activities were detected on blood agar plates.

2.4. Antibiogram pattern testing of Staphylococcus isolates:

The test was performed using standard agar disk diffusion method according to NCCLS, (2003). Nine commercial disks of different antibiotics commonly used as drug of choice in mastitis cases were used in this test. The antibiotics included; Ciprofloxacin (CIP) (5 µg), Flumequine (30 µg), Enrofloxacin (5 µg), Doxycycline (30 µg), Sulphamethoxazole-Trimethoprim (23.7 + 1.25 µg), Oxytetracycline (30 µg), Amoxicillin (10 µg), Erythromycin (15

µg). The degree of sensitivity was determined by measuring the clear zone of growth inhibition produced by diffusion of antibiotics from disc into the surrounding medium.

2.5.Molecular detection of *S. aureus*:

2.5.1. *S. aureus* DNA extraction:

DNA of isolated *S. aureus* was performed by using the boiled cell method according to (Sambrook et al., 1989).

2.5.2. PCR procedures:

clfA gene that is specific for coagulase positive *S. aureus* was detected by the aid of oligonucleotide primers set of the following sequence:

Gene	Primer sequence (5'-3')	Product size	Reference
*clf*A	GCAAAATCCAGCACAACAGGAAACGA	638bp	Mason et al., 2001
	CTTGATCTCCAGCCATAATTGGTGG		

The reaction was included in a total volume of 25 µl in 0.5 µl Eppendorf tube according to iNtRON´s Maxime PCR PreMix Kit as follow; 12.5 µl of 2x PCR Master mix Sol., 5 µl of template DNA, 1.25 µl of forward primer (20 pmol), 1.25 µl of reverse primer (20 pmol) and 5 µl PCR grade water. The tubes were placed into the thermal cycler that already programmed. The PCR cycle conditions were optimized for each primer set. Briefly, the PCR cycling conditions consisted of initial denaturation at 94° C for 5 min, followed by 30 cycles each of denaturation at 94° C for 30 s, annealing at the temperature optimal for each primer set for 45 s, and extension at 72° C for 45 s. At the end of cycling, the tubes were stored at - 20 ˚C until needed for electrophoresis.

2.5.3. Detection of PCR products (Sambrook et al., 1989):

PCR products were showed by electrophoresis on 1.5% agarose gel in Tris borate EDTA buffer that was stained with 0.5 µg/ml ethidium bromide. Specific amplicons were observed under ultraviolet trans-illumination, compared with the DNA ladder and photographed.

2.6.Statistical analysis:

It was made using Chi^2 test to examine the significance differences of the detection rate of antibodies among different groups studied according to SAS, (2014).

3. RESULTS AND DISCUSSION

Mastitis is the most common disease affecting dairy animals causing significant economic losses that result from reduction of milk quality and quantity, culling or mortality of severely affected animals that may reach high percentage in some times, in addition to the high costs of animal treatment that may exceed the cost of its culling (Bergonier et al., 2003). Subclinical mastitis alone is responsible for loss of more than 20% of total dairy production (Contreras et al., 2003), this type of mastitis is caused mainly by CNS. In addition, *S. aureus* infections are responsible for 35% of dairy industry losses (Abo-Shama, 2014).

The results of this study that was tabulated in Table (1) indicated the higher prevalence of subclinical mastitis in sheep farms (32.96%) comparing with (30.92%) in goat farms, that come in accordance with Gebrewahid et al., (2012) who concluded that sheep are more susceptible to subclinical mastitis than goats. They returned this difference to species variation which may control their resistance and susceptibility to mastitis. Also, this result lay within the range of worldwide prevalence of subclinical mastitis according to Olechnowicz and Jaśkowski (2014). On the other hands, Hall and Rycroft (2007) recorded higher prevalence values of SCM ranged from 33 to 42% in goats herds. While Gebrewahid et al., (2012) achieved lower values (28.1% and 18.03%) in sheep and goat farms, respectively.

Table (1): Prevalence of subclinical mastitis:

Species	No. of examined samples	Positive	%
Sheep	455	150	32.96
Goat	485	150	30.92

Table (2): Prevalence of *Staphylococci* in sheep and goats farms:

Source of samples	Sheep			Goats		
	No.	Positive	%	No.	Positive	%
Milk	150	39	26.0	150	51	34.0
Bedding	50	17	34.0	50	18	36.0
Water	50	12	24.0	50	11	22.0
Total	250	68	27.2	250	80	32.0
Chi^2 value		5.40**			4.25**	

** = Significant at (P < 0.01)

Table (3): Distribution of Staphylococci isolated from goats farms according to coagulase test:

Staphylococci	Milk (n=150)		Bedding (n=50)		Water (n=50)		Total (n=250)	
	No.	%	No.	%	No.	%	No.	%
Staph. aureus	29	19.33	11	22.0	2	4.0	42	16.8
CNS	22	14.67	7	14.0	9	18.0	38	15.2
Total	51	34.0	18	36.0	11	22.0	80	32.0
Chi^2 value				5.22**				

** = Significant at (P < 0.01)

CNS: Coagulase negative Staphylococci

Table (4): Distribution of Staphylococci isolated from sheep farms according to coagulase test:

Staphylococci	Milk (n=150)		Bedding (n=50)		Water (n=50)		Total (n=250)	
	No.	%	No.	%	No.	%	No.	%
Staph. aureus	21	14.0	6	12.0	4	8.0	31	12.4
CNS	18	12.0	11	22.0	8	16.0	37	14.8
Total	39	26.0	17	34.0	12	24.0	68	27.2
Chi^2 value				4.32**				

** = Significant at (P < 0.01)

Table (5): Results and indications of antibiogram test performed for *Staphylococci* isolates obtained from sheep and goats farms (n=10/ each)**:**

Staphylococci / Antimicrobial discs	*Staph. aureus*		CNS	
	Inhibition Zone	Sensitivity	Inhibition Zone	Sensitivity
Ciprofloxacin (CIP 5 µg)	3 mm	+	6 mm	++
Flumequine (30 µg)	2 mm	+	0 mm	-
Enrofloxacin (5 µg)	0 mm	-	6 mm	++
Doxycycline (30 µg)	4 mm	+	0 mm	-
Sulpha.-Trimethoprim (25 µg)	2 mm	+	0 mm	-
Oxytetracycline (30 µg)	0 mm	-	7 mm	+++
Amoxicillin (10 µg)	7 mm	++	10 mm	++++
Erythromycin (15 µg)	4 mm	++	10 mm	++++

- Resistance
+ Weakly sensitive
++ Moderately sensitive
+++ Quite sensitive
++++ Highly sensitive

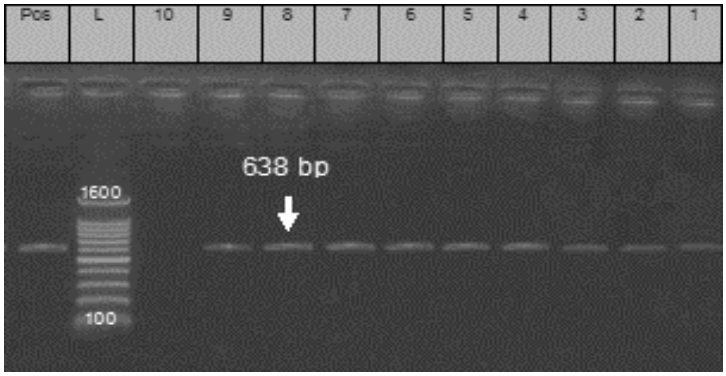

Photo (1): PCR products of *clf*A coding gene specific for coagulase positive *Staph. aureus* isolates obtained from different sources at sheep and goats farms. Lane Pos: Positive control, Lane L: 100 bp ladder, Lane 10: Negative control, Lanes 1, 2, 3, 4 and 5: Milk samples, Lanes 6 and 7: Bedding samples, Lanes 8 and 9: Water samples

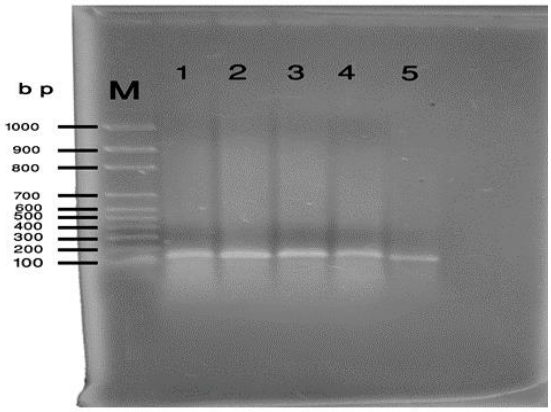

Photo (2): Agarose gel electrophoresis product of sea to sej TSST-1 (tst) genes of *Staph. aureus.* Lane M: 100 bp DNA marker; Lane 1-5: sea to sej TSST-1 (tst) genes. (173 bp); Lane 6: Negative control.

The results in tables (2 and 3) revealed that the higher isolation frequency of Staphylococcus species was recorded in goat's milk (34%), where *S. aureus* represented 19.33% while CNS share was 14.67%. these results come in violation of what is common, as it is well known that CNS are the most prevalent pathogens isolated from subclinical mastitis in small ruminants, the fact that was confirmed by many authors around the world like Lerondelle et al., (1992) who recorded 25% of subclinical mastitis cases in goats caused by Staphylococcus species in France, where *S. aureus* represented 2% and CNS prevalence was 23%. Also, Contreras et al., (2003) concluded that CNS makes up 44.7% to 95.9% of subclinical mastitis in dairy goats while *S. aureus* accounted for 4.1% to 18.0%. in addition, Hall and Rycroft, (2007) recorded 33% - 42% subclinical mastitis in three goat herds where CNS was the most prevalent organism (47%), while *S. aureus* come in the third orded (13%). Moreover, Marogna et al. (2012) detected mastitic infections in 22.7% of all goats in 31 farms where Staphylococcus species alone were detected in 73.5% of positive milk samples.

In sheep, the result revealed that the prevalence of Staphylococcus species was 26% where *S. aureus* isolation frequency was 14% while that of CNS was 12% (Tables 2 and 4). This result conflicted with the conclusion of Bergonier and Berthelot, (2003) that said that CNS are the principal causative agents of subclinical mastitis (30–95%), mainly in dairy ewes. Moreover, the current results were higher than that obtained by Marogna et al., (2010) who found that Staphylococcus species presented 20.7% of total

positive milk samples collected from 2198 udders of the Sarda sheep breed. On the other hand, Abo-Shama, (2014) estimated higher prevalence (30%) of *S. aureus* in raw milk samples of sheep. According to Gebrewahid et al., (2012) these variations returned to the variations in the breed, husbandry of the animals and agroclimatical conditions.

The obtained results also revealed the role of environment as a probable source of infection with Staphylococcus species, where table (3) showed prevalence of Staphylococcus in goat farms estimated by 36% for bedding samples (22% *S. aureus* and 14% CNS) and 22% for water samples (4% *S. aureus* and 18% CNS) while in sheep farms the prevalence of Staphylococcus species was 34% in bedding samples and 24% in water samples with higher prevalence of CNS 22% and 16% from bedding and water samples, respectively, while *S. aureus* recorded the lower prevalence values 12% and 18% from bedding and water samples, respectively (Table, 4). The role of animal pens in increasing the probability of Staphylococcus species was noticed by Albenzio et al., (2002) and Bergonier et al., (2003), they stressed on the importance of application of hygienic measures to control such infection.

Table (5) explained the results of antibiogram testing of Staphylococcus species isolated from goats and sheep farms. A complete resistance of *S. aureus* to Enrofloxacin and oxytetracyclin was noticed, while it showed only moderate sensitivity to Amoxicillin and Erythromycin antibiotics. These results were confirmed by Enright, (2003) who concluded that *S. aureus* frequently show multiple antimicrobial resistance patterns. Also, Abo-Shama, (2014) detected resistance of *S. aureus* to Erythromycin in sheep and goat raw milk followed by resistance to Amoxicillin-Clavulanic Acid, Ampicillin, Oxacillin and Penicillin G. in addition, Virdis et al, (2010) found that 56.0% of *S. aureus* were resistant to one or more antimicrobial agents where the highest resistance rates reported against kanamycin (28.0%), oxytetracycline (16.0%), and ampicillin (12.0%).

On the hand, CNS showed full resistant to Flumequine, Doxycyclin and sulphamethoprim but they were highly sensitive to Amoxicillin and Erythromycin. In conterary, Virdis et al, (2010) recorded 36.0% resistance of CNS to ampicillin that is an equivalent to Ampicillin. The resistance and susceptibility patterns of bacteria were studied by Waage et al., (2002) who concluded that the prevalence of antibiotic resistance usually varies between isolates from the different sampled stations and even between isolates from different herds on the same farm.

*clf*A is one of clumping factors belong to *S. aureus* that help it in colonization and establishment of infections Salasia et al., (2004) and its detection using PCR used for genotypic characterization of isolated *S. aureus* (Abo-Shama, 2014). Photo (1) confirmed the PCR products of *clf*A coding gene specific for coagulase positive *S. aureus* isolates obtained from different sources at sheep and goats farms.

Finally, the results obtained from this study suggested that sheep were more susceptible to subclinical mastitis than goats, although goats were more liable to Staphylococcus infections than sheep. Also, *S. aureus* played the main role in induction of subclinical infections in both goats and sheep. In addition, animal pens may act as reservoirs and source of Staphylococcus infection. At last, it is obvious that resistance of Staphylococcus species to different antibiotics become increased, what is imperative to reconsider the ways in which we use these antibiotics.

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