Some bacteriological and genetical studies on Staphylococcus species isolated from buffaloes milk

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Abstract

This study is concerned with coagulase negative *Staphylococci* (CoNS) isolated from clinical and subclinical mastitis of buffaloes in Giza, Kalubia and Monofia governorates. From the total examined dairy buffaloes (100), there were 62 cases of mastitis which were classified into clinical and subclinical cases. A total of 365 individual quarter milk samples were collected from mastitic and apparently healthy normal udders, from which 21 quarter milk samples from clinically infected *animals* (14 buffaloes) and 192 quarter milk samples from subclinical cases (48 buffaloes). The incidence of bacteria was 37.5% from clinical cases, but the bacterial incidence in subclinical mastitis was 44.8%. The incidence of positive samples yielding *Staphylococci* was 21.1% totally where 52.4% for clinical cases and 17.7% for subclinical cases. The incidence of *S.aureus* was 33.3% and 9.9% for clinical and subclinical cases respectively. While, the incidence of *S.epidermidis* was 19% and 7.8% for clinical and subclinical cases respectively. The incidence of coagulase positive and coagulas negative for*, S.aureus* was 80.8 %, 19.2 % and 100%, 0.0% for *S.epidermidis* respectively. All CoNS (5 isolates) were considered as resistant to 1 m oxacillin while the coagulase positive *Staphylococci* were considered as oxacillin susceptible. PCR was applied on 5 CoNSA for the presence of mecA gene. Since the isolates which revealed the presence of mecA gene considered as methicillin resistant.

Key words: *Staphylococci , subclinical mastitis, buffaloes, S. aureus, oxacillin, methicillin .*

Introduction

Staphylococcus aureus is a major pathogen of bovine mastitis. worldwide. Despite implementing intensive control measures it is difficult to eradicate the intramammary infection caused by this pathogen and it remains, a substantial economic problem, **(Salmon, 2002).** Coagulase – negative *Staphylococci* (CoNS) are mainly involved in the inflammatory processes of the bovine udder. Mastitis represents the economically most important disease in the dairy industry worldwide **(Aarestrup and Schwarz 2006).** However, during recent years, CoNS have become the most common bovine mastitis isolates in many countries and are regarded as emerging mastitis pathogens **(Pyorala and Taponen**, **2009)**.

Antimicrobial agents are widely used in the treatment of udder infections. Among the antimicrobial agents approved for use in bovine mastitis, β-lactams, such as penicillin and cephalosporins, play a key role. Resistance to β -lactams in *Staphylococci* is mediated by either β-lactamases of the Blaz type or the mecA - encoded alternative penicillin – binding protein PBP2a, which shows a reduced binding to all β -lactam antibiotics currently available for mastitis therapy. Strains expressing the mecA gene are referred to as methicillin resistant **(Aarestrup and Schwarz, 2006).**

The aim of the present investigation is directed to throw the light on the following:

- Isolation and identification of *Staphylococcus* species from milk samples collected from apparently healthy and mastitic buffaloes.
- Detection of coagulase positive and coagulase negative staphylococcus isolates.
- Testing the sensitivity of coagulase negative *Staphylococcus* isolates to oxacilline.
- Amplification of the (mecA) gene in a PCR based assay for rapid detection of resistance

gene responsible for methicillin resistant *Staphylococcus* isolates.

Material and Methods

Milk Samples:

A total of 100 dairy buffaloes in 3 herds form different farms were examined for mastitis.
According to clinical observation and California mastitis test **(Schalm *et al*., 1971)**, the mastitic cases (62) were classified into clinical and subclinical cases. A total of 365 individual quarter milk samples were collected from mastitic and apparently healthy normal udders, and they were divided as follow: 21quarter milk samples form clinically infected animals (14 buffaloes), 192 quarter milk samples form subclinical cases (48 buffaloes) and 152 quarter milk samples from healthy normal udders (38 buffaloes). Samples were collected in sterile macCarteny and they were sent to laboratory in an ice box with a minimum of delay.

Isolation and identification of *Staphylococcus* species:

According to **Quinn *et al*., (2002)**. Milk samples were incubated aerobically at 37°C for 24 hours, then centrifuged at 3000 r.p.m. for 20 minutes. The cream and supernatant were discarded. A loopful from the sediment was streaked onto the surface of sheep blood agar, nutrient agar, Mannitol slat agar and Baird parker medium. The inoculated plates were incubated at 37°C for 24-48h then the suspected colonies were identified morphologically, Gram staining and biochemically using API kit system (Biomeraux france).

Coagulase test:

It was applied according to **Quinn *et al*., (2002)**, applying slide coagulase test and tube coagulase test.

Sensitivity of coagulase negative *Staphylococcus* (CoNS) isolates to oxacillin:

It was done by using disc diffusion standard technique according to **Finegold and Martin (1982)** using 1 mg oxacillin disc. The test was done on 8 coagulase negative isolates and also, on 5 coagulase positive isolates as control.

Polymerase Chain reaction (PCR) for detection of (mecA) gene:

It was done according to **Sritharan and Barker (1991)** and **Riffon *et al*. (2001)** on oxacillin resistant isolates for the presence of (mec A) gene.

Table (1) : The primers used for PCR.

Primer	Sequence	Product size (bp)
Mec A R1	GTGGAATTGGCCAATACAGG	1339
Mec A R 2	TGAGTTCTGCAGTACCGGAT	

Table (2): Prevalence of bacteria causing clinical and subclinical mastitis among buffaloes.

Total number of examined animals	Number of healthy animals	Number of mastitic animals	%	Type of mastitis									
				Clinical					Subclinical				
				No. of animals	*%	No. of Quarters	+ ve Q	**%	No. of animals	*%	No. of Quarters	+ ve Q	**%
100	38	62	62	14	14	56	21	37.5	48	48	192	86	44.8

Q = quarters.
* Percent was calculated according to total number of examined animals.
** Percent was calculated according to number of quarters.

Table (3): Prevalence of *Staphylococcus* species isolated from clinically and subclinically mastitic milk samples in buffaloes.

Source of milk samples	No. of examined milk samples	*Staphylococcus* species				Total number of isolates	*%
		S.aureus		*S.Epidermidis*			
		No.	*%	No.	*%		
Clinical mastitis	21	7	33.3	4	19	11	52.4
Subclinical mastitis	192	19	9.9	15	7.8	34	17.7
Total	213	26	12.2	19	8.9	45	21.1

No. : Positive number.
* The percentage was calculated according to the number of examined milk samples.

Table (4): Prevalence of coagulase positive and coagulase negative *Staphylococci* (CoNS) isolates.

Type of isolates	*S. aureus*		*S. epidermidis*	
	No	%	No	%
Total No. of examined isolates	26	100	19	100
Coagulase + ve isolates	21	80.8	19	100
Coagulase - ve isolates	5	19.2	0.0	0.0

No. = number.
* The percentage was calculated according to the total number of examined isolates.

All coagulase negative *Staphylococci* (CoNS) (5 isolates) were resistant to 1 m oxacillin since it gave zone diameters of ≤17mm around 1mg oxacillin disc. While, the coagulase positive islolates gave zone diameters of 18-23mm and they were considered as oxacillin susceptible.

Results of PCR for detection of (mecA) gene on 5 CoNS isolates. 3 isolates revealed the presence of (mecA) gene (methicillin resistant) while 2 isolates were negative to the presence of (mecA) gene (methicillin susceptible). **Pho-**

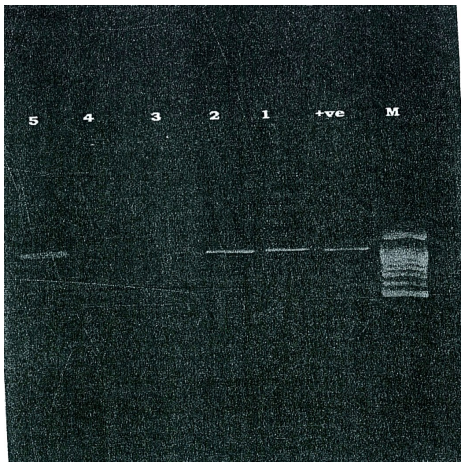

Photo.(1): Agarose gel electrophoresis showing amplification of the 1339 bp fragment of mecA gene
M: The DNA molecular weight marker (100bp ladder)
+ve: positive control
Lane (1,2&5): methicillin resistant *Staphylococcus aureus*.
Lane (3&4): methicillin sensitive *Staphylococcus aureus*.

Discussion

Bovine mastitis is the most common infectious disease affecting dairy animals and remains the most economically important disease of dairy industries around the world. Dairy producers losses are primarily due to lower milk yields, reduced milk quality and higher production costs or even destruction of the mammary gland **(Phuektes *et al*., 2001)**. Because of the organisms propensity to acquire antimicrobial resistance, where as most infections can be treated or prophylacted with antibiotic; antimicrobial resistance of *S.aureus* especially methicillin resistant *S.aureus* (MRSA) continues to be a problem for clinicians worldwide **(Shittu and Lin, 2006).** Coagulases negative *Staphylococci* (CoNS) are mainly involved in inflammatory process of the bovine udder **(Andrea *et al*., 2010)**. The data presented in Table (2) showed the examination of 86 apparently healthy buffaloes, depending on CMT according to **Schalm *et al*., (1971)** revealed that subclinical mastitis was recovered from 48 buffaloes with an incidence of 44.8%. This percentage seems to be high and confirm the hypothesis of **National Mastitis Council (1997)** which stated that each case of clinical mastitis in dairy farm, may be associated with about 15-20 cases of subclinical mastitis. On the other hand, this finding, is higher than that recorded by **Hussian *et al*., (1984)** as they recorded an incidence of subclinical mastitis was 19% between buffaloes. The results revealed that, clinical mastitis was recovered from 14 buffaloes with an incidence of 37.5%. In this concern, **El -Khodery and hoedemaker (2005)** recorded that the prevalence of bacteria causing clinical mastitis was 69.74%. while, **Refai *et al*., (2005)** recorded that the prevalence of bacteria causing clinical mastitis was 87% and that caused subclinical mastitis was 48.7%. The results presented in Table (3) showed the prevalence of *Staphylococcus* species isolated from clinically and subclinically mastitic milk samples in buffaloes, where *S.aureus* was isolated with an incidence of 33.3% from clinical mastitis and 9.9% from subclinical mastitis.

Approximately similar incidence (22.5%) of *S.aureus* in clinical mastitis was reported by **Silva (1996).** In subclinical mastitis, a similar incidence of *S.aurerus* was reported by Aki (2000). On the other hand, *S.epidermidis* was isolated from clinical and subclinical mastitis with an incidence of 19% and 7.8% respectively. In this concern, **Refai *et al*., (2005)** who recovered *S.aureus* from 23.7% and 6.3%, they also isolated *S.epidermidis* in 6.5% and 5.8% from clinical and subclinical mastitic cases respectively. The results in Table (4) showed the prevalence of coagulase positive and coagulase negative *Staphylococci* (CoNS) isolates.

The incidence of coagulase positive and coagulase negative *S.aureus* isolates was 80.8% and 19.2% respectively, while the incidence of coagulase positive and coagulase negative *S.epidermidis* isolates was 100% and 0.0% respectively. In this concern, **Capita *et al*., (2002)** mentioned that 21.9% strains showed clot formation (coagluase positive) within 2 hrs and 100% strains formed firm clots after 6 hrs at 37°C and **Andrea *et al*., (2010)** who isolated 121 CoNS isolates from individual cases of bovine mastitis.

In the present work all coagulase negative *Staphylococcus aureus* (CoNSA) (5 isolates) were resistant to 1m oxacillin. Since oxacillin maintains its activity during storage better than methicillin, laboratory diagnosis of methicillin resistance is based on the testing of oxacillin. According to recommendations of the CLSI, oxacillin – resistant *Staphylococcus* isolates shall be reported as resistant to other β-lactam antibiotics. Because of these implications for the choice of antimicrobial agents for therapeutic applications, it is of major importance to assess oxacillin resistance correctly **(CLSI. 2008).**

Results of PCR revealed the positive amplification of the 1339 bp fragment of mecA gene from the extracted DNA of 3 coagulase negative *S.aureus* (CoNS) isolates out of 5 examined CoNS. In this concern, **Andrea *et al*., (2010)** stated that the mecA gene was identified in 15 of 16 (CoNS) oxacillin resistant isolates, the single mecA -negative resistant isolate had an oxacillin zone diameter of 16mm,

which classified this isolate as borderline resistant. Also, **Van Duijkeren *et al*., (2004)** reported that methicillin resistance in *Staphylococci* is mediated by the mecA gene, encoding the penicillin – binding protein 2a (PBP2a), which has a reduced affinity for the penicillinase – resistant penicillins like methicillin and oxacillin and for all other β-lactam antibiotics. This meaning was supported by **Strommenger *et al*., (2006)** who mentioned that all isolated *S.aureus* which carrying the mecA gene mediated resistance to β-lactam antibioltics. Thus, applying PCR for the presence of mecA gene, this test may prevent potentially efficacious cephalosporins from being excluded from mastitis therapy if the phenotypically oxacillin – resistant CoNS are mecA-negative.

Conclusion

Incidence of subclinical mastitis is high in the examined buffaloes. The results of this study confirmed that the currently available oxacillin breakpoints are not able to correctly differentiate between mecA- positive and mecA- negative CoNS isolates. The application of additional test, such as mec A PCR, may help to reliably detect the presence of the mecA gene in questionable isolates. An important point must be taken in attention is the avoidance of bad hygiene in dairy farms, since *Staphylococci* can be transmitted easily from animal to animal by contaminated common udder wash clothes, residual milk in teat cups and milking equipments .

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بعض الدراسات البكتريولوجية والجينية على الميكروبات المكورة العنقودية المعزولة من ألبان للجاموس

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الملخص العربى

تم اجراء هذه الدراسة على الميكروبات المكورة العنقودية السالبة لاختبار الكواجيولاز والمعزولة من الجاموس المصاب بالتهاب الضرع الظاهر والخفي.

تم تجميع عينات لبن من عدد 100 جاموسه حلاب وكان منها عدد 62 حالة التهاب ضرع (ظاهر وخفي). تم تجميع عدد 365 عينة لبن (عينة من كل ربع ضرع) من المائة جاموسه موضوع البحث على النحو التالي:

21 عينة لبن من الحيوانات المصابة بالتهاب الضرع الظاهر وكان عددها 14 جاموسة، 192 عينة لبن من الحالات المصابة بالتهاب الضرع الخفي وكان عددها 48 جاموسة. وكانت نسبة عزل البكتريا من الحيوانات المصابة بالتهاب الضرع الظاهر 37.5% بينما كانت 44.8% من الحيوانات المصابة بالتهاب الضرع الخفي. وكانت نسبة وجود الميكروب المكور العنقودي 21.1% من حالتي التهاب الضرع بينما كانت نسبة وجود الميكروب المكور العنقودي 52.4% ، 17.7% من حالات التهاب الضرع الظاهر والخفي على التوالي. وكانت نسبة وجود الميكروب العنقودى الذهبي 33.3%، 9.9% من حالات التهاب الضرع الظاهر والخفي على التوالي. بينما كانت نسبة وجود الميكروب العنقودي إبيدرميدس 19%، 7.8% من حالات التهاب الضرع الظاهر والخفي على التوالي.

وكانت نسبة وجود الميكروبات موجبة الكواجيولاز والسالبة كواجيولاز للميكروب العنقودى الذهبي هي 80.8%، 19.2 % وكانت 100%، 0.0% للميكروب العنقودي إبيدرميدز على التوالي.

وكانت كل الميكروبات العنقودية الذهبية السالبة لإختبار الكواجيولاز (5 ميكروبات) مقاومة لاختبار الحساسية بالأوكساسيلين بينما كانت كل الميكروبات العنقودية الموجبة لإختبار الكواجيولاز حساسة للأوكساسيلين و بإجراء اختبار البلمره المتسلسل على عدد 5 معزولات من الميكروب العنقودى الذهبي السالبة لاختبار الكواجيولاز لمعرفة وجود macA جين والمسئول عن المقاومة للمثيسلين حيث أن المعزولات التى تحتوى على هذا الجين تعتبر مقاومة للمثيسلين.