Prevalence of coagulase (coa) gene and mec A gene of *S. aureus* isolated from bovine clinical mastitis

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ABSTRACT

S. aureus is recognized worldwide as one of the frequent cause of intramammary infections in dairy cows. In order to determine the prevalence of ***S.aureus*** bovine clinical mastitis as well as detection of coagulase and mec A genes of ***S,aureus*** , a total of 78 quarter milk samples were collected from clinical cases of mastitis after the investigation of 100 lactating Friesian cows at Ismailia Governorate. By bacteriological examination the percentage of ***S.aureus*** in bovine clinical mastitis was (28.2%). Ten isolates were subjected to PCR for detection of coagulase gene (coa) gene, (100%) were positive. PCR protocol used for amplification and detection of (mec A) gene of MRSA as a confirm diagnosis of their resistance to methicillin antibiotic . All MRSA isolates were 100% positive . Finally, the prevalence of MRSA mec A gene was very low in comparison with coagulase gene.

INTRODUCTION

S.aureus is one of the common causes of mastitis worldwide, which is of economic importance to the dairy industry . Raw milk is a potential source of ***S.aureus*** in milk and milk products, especially in the case of defective pasteurization. The main reservoir of ***S.aureus*** seems to be the infected quarter. Molecular epidemiological analysis of the bovine S.aureus population suggested that a small number of clonal types were responsible for most infections, and that strains had a broad geographic distribution. The organism has a capacity to produce a large number of putative virulence factors . Some of these factors may be of more importance than others in different diseases or at different stages of the pathogenesis of particular infections, as not all factors are produced by each strain. ***Kalorey et al (2007)***

Coagulase is one of the most important virulence factors, which is an exoenzyme and clots plasma by conformational activation of prothrombin ***(Panizzi et al, 2004).***

The transmission of bovine MRSA to humans is possible and may contribute to outbreaks in animal

and human populations ***(Lee, 2003).*** MRSA strains show pathogenic and epidemiological characteristics in various ways such as mutation, clonal evolution ***(Fitzgerald et al, 2001)*** and horizontal gene transfer ***(Brody et al, 2008).*** These evolutionary processes enhance the pathogenic and antimicrobial-resistant properties of ***S. aureus*** strains. However, a limited diversity of ***S. aureus*** strains or clones cause most of the mastitic infections in each geographical region, as these isolates are better adapted to infect animals ***(Moon et al, 2007).*** Identification and early elimination of pathogenic MRSA strains at the herd level is possible by the use of different molecular microbiology tools. ***Kumar et al (2011)***

Hence, it is necessary to know which endemic strains of ***S. aureus*** in dairy cattle populations are highly pathogenic and methicillin-resistant.

The objective of this study was to determine the prevalence of ***S.aureus*** bovine clinical mastitis , detection of MRSA strain by disc diffusion antimicrobial susceptibility and detection of ***S.aureus*** Coagulase and methicillin resistant genes .

MATERIAL AND METHODS

Milk samples

A total of 78 milk samples were collected from clinical cases of mastitis after the investigation of 100 lactating Friesian cows(400 quarters) at Ismailia Governorate.

Isolation and identification of *S.aureus*:

Milk samples were incubated aerobically for 24 hours at 37 °C to achieve potential bacterial growth and a loopfull was taken from each sample and streaked onto nutrient agar, blood agar and Mannitol salt agar plates . All plates were incubated at 37°C for 24-48 hours and examined for bacterial growth. Bacterial colonies were identified morphologically using Gram's stain as well as biochemical methods described by ***(Quinn et al, 1994).***

Antimicrobial susceptibility testing by disc diffusion method:

The susceptibility to methicillin antibiotic was tested according to the procedures of ***NCCLS (2007)*** using disc diffusion technique. The susceptibility of the strains was determined according to the size of inhibition zone.

PCR detection of (coa) gene and (mec) gene of *S.aureus*

Ten isolates were subjected to PCR for detection of coa gene and mec gene ,(4 MRSA strains and 3 MSSA strains from isolated from cases of clinical mastitis, one human MRSA strain and 3 MRSA strains previously isolated from cases of subclinical mastitis)

1-Extraction of DNA from ***S.aureus*** isolates by boiling method according to **Van Eys et al.(1989)**

2-Polymerase chain reaction:

DNA samples were tested [in 50 µl. Reaction volume in a 0.2 ml. PCR tube , containing PCR buffer] (50

mM Kcl , 10 mM tris - Hcl , 1mM $Mgcl_2$) each dNTPS (Deoxy nucleotide Triphosphate) 200 uM each (dATP , dGTP , dCTP and dTTP) , [Two primer pairs each at 50 picomol / reaction] and 0.5 of taq DNA polymerase . Thermal cycling in a programmable heating block (Coy vorporation, Grasslake, Michan, USA) was done. A negative control PCR reaction with no template also was included in this assay. MRSA gene primers were prepared as described by ***Vannuffel et al (1995)*** while coagulase gene primers were synthesized as described by ***(Hookey et al., 1998).***

Table (1): *list of primers used for PCR assay*

Primer	Primer Sequence.	Molecular weight (bp)	Annealing temp.
Coa 1	**ATA GAG ATG CTG GTA CAG G**	**600**	**58°c**
Coa 2	**GCT TCC GAT TGT TCG ATG C**		
MecA-1	**TGGCTATCGTGTCACAATCG**	**310**	**66°c**
MecA-2	**CTGGAACTTGTTGAGCAGAG**		

PCR Protocol: Initial Denaturation at 94 ^{o}C for 4 min, **Denaturation** at 94 ^{o}C for 1 min, **Annealing** at 58 ^{o}C for coa primer and 66 ^{o}C for (mec) gene primer for 1 min, **Extension** at 72 ^{o}C for 1 min . Cycles repeated for 39 times and proceeded by initial denaturation at 95 ^{o}C for 5 min. and followed by final extension at 72 for 10 min.

3-Screening of PCR products: ten µl of amplified PCR product was analyzed by electrophoresis on a 2% agarose gel stained with 0.5 µg of ethedium bromide / ml. Electrophoresis was carried out in 1X TAE buffer at 80 volt for 1 hour. The gel was photographed in order to obtain a permanent record using UVP BioSpectrum Imaging Systems, UVP® LLC.

RESULTS

Table(2): *Prevalence of apparently normal samples and clinically mastitic milk samples of total collected milk samples.*

No. of examined animal (Friesian cow)	No. of milk samples	No. of Apparently normal milk samples	% of Apparently normal milk samples	No. of clinical mastitis milk samples	% of clinical mastitis milk samples
100	400	322	80.5 %	78	19.5 %

Table (3): *The percentage of S.aureus bovine clinical mastitis*

Total clinical mastitis milk samples	No. of +ve samples of *S.aureus*	% of +ve samples of *S.aureus*	No. of –ve samples	% of –ve samples
78	22	28.2%	56	71.79%

Table (4) : *Prevalence of Methicillin resistant S.aureus (MRSA) and Methicillin sensitive S.aureus (MSSA)*

No of *S.aureus* isolates	No. of MRSA	% of MRSA	No. of MSSA	% of MSSA
22	4	18.18 %	18	91.82%

Table (5): *Prevalence of Coa gene in isolated S. aureus*

10 isolates from 22 clinical mastitis isolates				
No. of samples subjected to PCR for Coa gene detection	**No. of +ve Coa gene**	**% of +ve Coa gene**	**No.-ve Coa gene**	**% of .-ve Coa gene**
10 isolates from 22	10 isolates	100%	0	0%

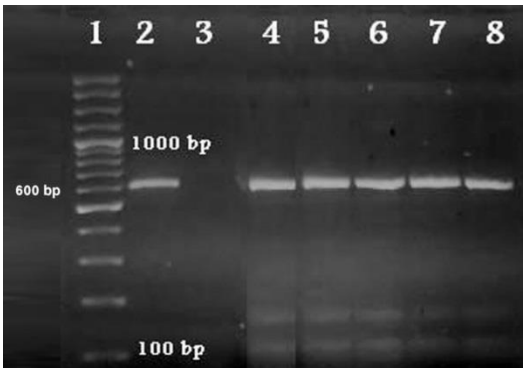

Figure (1) *illustrated the positive amplification of 600 bp fragment of Coa gene from the extracted DNA of isolated S.aureus .*

- Lane 1:(100 bp DNA ladder).
- Lane 2: control coagulase positive ***S.aureus*** strain according to ***(EL-Gammal, 2011)***
- Lane 3: control negative.
- Lanes 4-8: showed coagulase positive isolated ***S.aureus.*** from clinical mastitis milk samples.

Table (6)*: Prevalence of mec A gene in isolated S . aureus*

4 isolates from 22 clinical mastitis isolates				
No. of samples subjected to PCR for mec A gene detection	**No. of +ve mec A gene**	**% of +ve mec A gene**	**No.-ve mec A gene**	**% of .-ve mec A gene**
4 isolates from 22	4 isolates	100%	-	-

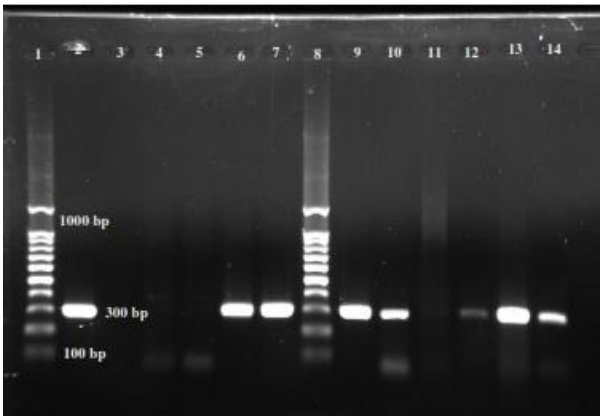

Figure (2 *) illustrated the positive amplification of 300 bp fragment of MRSA (mec A) gene from the extracted DNA of S.aureus isolates.*

Lanes 1 and 8 : (100 bp DNA ladder)
Lanes 2, 6, 7 and 9: +ve isolate for (mec A) gene (clinical mastitis isolate) With specific band 300(bp)
Lane 3: controle negative
Lanes 4, 5 and 11: -ve isolate for (mec A) gene
Lane 10: +ve MRSA human isolate for (mec A) gene (Isolated from Suez canal university hospital)
Lanes 12, 13 and 14: +ve isolates for (mec A) gene (subclinical mastitis isolates)
With specific band 300(bp) ***(El Gammal, 2011)***

DISCUSSION

In the present work, after the investigation of 100 lactating Friesian cows for the udder inflammatory signs as pain , hotness and the presence of bloody milk, the percentage of clinical mastitis was (19.5%.) as shown in Table (2) and this agree with the finding of ***Petrovski et al (2009)*** this may be due to the case hygienic measures and disagreed with the finding of ***Gianneechini et al (2002)*** . It seems that in Egypt there is high incidence of clinical mastitis compared to their finding , the high sample size and may be the herd area explains that low level of clinical mastitis in their situation .

As shown in Table (3) , the bacteriological examination of the collected samples revealed that 22 (28.2%) samples were positive for ***S.aureus*** and the other 56 (71.79%) samples were positive for other types of bacteria . These results agreed with ***(Bedane et al, 2012)*** which reported that ***S.aureus*** is responsible for approximately 30% to 40% of all mastitis cases. while ***(Scherrer et al, 2004)*** recorded that the high incidence of ***S.aureus*** mastitis is due to its transmission usually occurs during the milking

process by milker's hands, udder cloths or sponges and the milking machine. When milk from infected cows contaminates the teat cup liners and transfers the infection to the next animal milked with that unit. So, serious herd infections are frequently related to problems of mechanical milking and improper milking hygiene ***(Asperger and Zangerl, 2003)***

As shown in Table (4), the percentage of Methicillin resistant ***S.aureus*** (MRSA) from 22 ***S.aureus*** isolates was 18.18% (4 isolates). This finding seems to be in agreement with those of ***(Hanselman et al , 2006)*** . Resistance to methicillin occurred mainly due to the presence of mecA gene on ***S.aureus*** chromosome that responsible for the production of Penicillin binding protein $PBP_{2a.}$ ***(Ito et al, 2003)***

As shown in Table (5) and Fig. (1) , PCR protocol used for amplification and detection of coagulase (coa) gene of ***S.aureus*** isolates to confirm their pathogenicity as the presence of coagulase gene is an index of virulence . Ten isolates from 22 clinical mastitis milk specimens were subjected to PCR for detection of (coa) gene and all (100%) were +ve. In this work the amplification of coagulase gene resulted in a single amplicon, indicating no size polymorphisms of this gene, this agreed with that obtained by ***(Stephan et al ,2001)***,. However, the coagulase gene amplification in other studies ***(Scherrer et al, 2004)*** resulted in different amplicons, indicating coagulase gene size polymorphism. At present, no information is available about the sequence variation of these strains.

As shown in Table (6) and Fig. (2), PCR protocol used for amplification and detection of MRSA (mec A) gene as a confirm diagnosis of their resistance to Methicillin . All 4 MRSA strains were positive for mec A gene. One human MRSA strain was used as a +ve control. The obtained results of *mec*A PCR disagrees with ***(Catherine et al , 2003)*** as they found 12 strains could be phenotypically classified as MRSA out of 811 strains of ***S. aureus*** , but all examined strains were *mec*A negative. The advancing in PCR technology may explain this variations and they mentioned that ***S. aureus*** is recognized as a major contagious mastitis agent worldwide, but this does not seem to be true of MRSA . Also this finding disagrees with the finding of ***Guerin et al (2003)*** who after analyzing of 119 isolates of ***S. aureus*** collected between 1998 and 2000 in France from cows with clinical mastitis and no MRSA was found.

In conclusion, PCR is a rapid and specific diagnostic method used for genetic characterization of bovine ***S.aureus.*** The prevalence of coagulase gene among ***S. aureus*** isolates was high in comparison with the prevalence of mec A gene of MRSA. The differentiation in prevalence rates of MRSA findings

may be explained by different antimicrobial national policies and regulations. To make possible an accurate comparison of antimicrobial policies, national public health institutions (human and veterinarian) should annually collect data on kilograms of active antimicrobial agents used by species, route of administration and purpose of use (therapeutic, prophylaxis or growth promotion).

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تعيين وجود جين (coa) وجين mec A فى بكتيريا المكور العنقودي الذهبي المعزولة من التهاب الضرع الاكلينيكى فى الماشية

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بكتيريا المكور العنقودى الذهبى يعتبر من الميكروبات المسببة لالتهاب الضرع فى الماشية حول العالم. فى هذا البحث تم تعيين وجود بكتيريا المكور العنقودى الذهبى فى ٧٨ عينة لبن معزولة من حالات التهاب الضرع الاكلينيكى وتعيين وجود جين coagulase و جين mec A فى هذه العينات . بعد اجراءات الاختبارات البكتريولوجية لعينات اللبن كانت نسبة وجود بكتيريا المكور العنقودى الذهبى هى 28.2%. وتم استخدام اختبار البلمرة المتسلسل بتحديد جين (coagulase) لعدد ١٠ عينات وكانت كلها ايجابية بنسبة (١٠٠٪) وهى دليل على ضراوة البكتيريا المعزولة . واستخدام اختبار البلمرة المتسلسل بتحديد جين (mec A) الخاص بنوع الميكروب المكور العنقودى الذهبى (MRSA) لعدد العينات التى كانت مقاومة للمضاد الحيوى الميثيسيللين وكانت كلها ايجابية بنسبة (١٠٠٪) . وفى هذه النتائج تبين ان نسبة وجود جين (mec A) اقل بالمقارنة بنسبة وجود جين (coagulase).