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SEQUENCING AND PHYLOGENETIC CHARACTERIZATION OF S. AUREUS THERMONUCLEASE GENE

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ABSTRACT

Mastitis is considered the most predominant production infectious disease in dairy industry all over the world including Egypt, The difficulty of mastitis occurs due to its chronic nature of the disease, extremely economic losses, extensively spread all over the year and intense decrease in the milk production. *Staphylococcus aureus* is one of the most common bacterial agents causing mastitis and having public health importance. A total of 248 milk samples were collected from different dairy farms, 77 samples from mastitic milk, 171 from apparently normal milk. 24 samples were positive for *S. aureus* by applying coagulase test. This study is concerned with the detection of thermonuclease (NUC gene) of *S. aureus* through PCR reaction, sequencing and phylogenetic analysis. Sequence alignments and phylogenetic analysis revealed high level of similarities between the different alleles isolated from different samples. Genotyping and sequencing analysis of NUC gene is a good tool to understand the relationships and differences between different isolates of *S. aureus* causing mastitis in dairy farms.

Key words: *NUC gene, S. aureus, mastitis, Sequencing and Phylogene nesis.*

INTRODUCTION

Mastitis is considered the most predominant infectious disease causing economic losses in dairy industry all over the world. (Seegers *et al.*, 2003 and Petrovski *et al.*, 2006). The effective consequences of mastitis occur due to chronic nature of the disease, high economic losses, widely spread all over the year and dramatic decrease the milk productivity. (Sudhan and Sharma, 2010).

Bacterial species are the most common pathogenic agents causing mastitis in bovine. The reports indicates more than 137 microbes are the etiological agents of mastitis. (Watts, 1988), belongingto a wide variety of bacteria, mycoplasma, yeast and fungi. The staphylococci is considered one of the most effective species whichinducing mastitis (Giesecke *et al.*, 1994).

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S. aureus causes intramammary infection through the teat orifice, breaching the streak canal, and entering the mammary gland. The adherence of the bacterium to the epithelial cells of the mammary gland make it difficult to be washed away. In addition, it secretes different factors through which decreasing the phagocytic activity like protein A, capsuleand pseudo capsule (Sutra and Poutrel, 1994).

Staphylococcus aureus infection is characterized by intermittent shedding from the infected udder and consequently the bacteriologically negative results of the examined milk may do not guarantee that the animal is free from infection (Radostitis *et al.*, 2000).

There are a number of methods such as polymerase chain reaction (PCR) that have been used for this purpose. PCR has proven to be a powerful research tool, and its use for the sensitive and specific detection of microorganisms and antibiotic resistance genes is increasing in clinical microbiology laboratories. PCR has been used to amplify a segment of the nuc gene that is specific for *S. aureus* (Khan *et al.*, 2007 and Zahan *et al.*, 2009).

MATERIALS and METHODS

Sampling:
A total of 248 milk samples (mastitic and apparently normal) from dairy cows belonging to 3 Egyptian governorates (Qena, Damietta and Al-Bouhaira) 80, 139 and 29 and the number of mastitic and apparently healthy cattle in the 3 groups are 18 -62, 59-80 and 0-29 respectively. All samples forming the first and second group were collected from established dairy farms while the last group were collected from solitary home raising dairy cattle.

The milk samples were collected in sterile single use disposable plastic falcon tubes with tightly fitted caps (15 ml), all the sanitary measurement used routinely in milk samples collection were adopted and all collected samples were frozen immediately at -20ºC (Pamela, 2005)

Methods:
Isolation and culturing of *S. aureus*:
The frozen samples were thawed at the room temperature for 4-6 hrs and thoroughly mixed with vortex till complete homogenization. The preparation of baired-parker media and pouring into sterile petri-dishes (9 cm diameter) followed according to the method (Baired-parker, 1962). 3 typical and large colonies was harvested and picked up by a sterile metal bacteriological loop and then immersed in the glycerol stock and kept immediately at -70 to -80 ºC (Feltham *et al.,* 1978 ; Jones *et al.,* 1991).

Biochemical tests:
The coagulase test was performed by two different methods; the slide coagulase test and tube coagulase test (Cookson, 1997 and Wichelhaus *et al.,* 1999).

Preparation of PCR reaction of NUC gene:
DNA preparation from bacterial culture (McLauchlin *et al.,* 2000):
Picking up one or two of pure colonies of overnight growth cultures on Blood agar and melted with 40 ul water (DNase free water) and heated for 5 minutes at 95 ºC in a thermocycler.

Oligonucleotide primers
Forward and reverse primers of NUC gene (100pmol/ul, eurofins MWG Operon),. were diluted in sterile water (Invitrogen TM, Carlsbad, CA) to make a final concentration 20pmol/ul for each primer; SA_nuc (F)5′GCGATTGATGCTGATACGGTT′3 and SA_nuc(R)5′ AGCCAAGCCTTGA CGAACTAAA ′3 (Brakstad *et al.,* 1992).

Procedures:
PCR products of NUC gene were performed using microbial DNA, Taq polymerase (Qiagen) and Master Mix solution. PCR master mix was prepared in a PCR tubes on ice (25 µl of PCR mixture); samples were placed in a thermocycler (Gene Amp- PCR system 2700 Version 2.0- Bio systems A&B). The PCR conditions were adjusted according to NUC gene after optimization trials to reach the best melting temperature. Amplification conditions were denaturation for 3 min at 94ºC, followed by 30 cycles of 94ºC for 1 min, 58ºC for 1 min and 72ºC for 1 min, with final extension at 72ºC for 5 min. The PCR products were analysed by 2 % agarose gel electrophoresis (Seakem® LE Agarose, Cambrex Bioscience, Rockland, Inc. Rockland, ME USA). PCR products (267 bp) were purified following the QIAquick PCR Purification kit protocol (QIAGEN) and PCR fragments were chemically labelled with fluorescent dyes to be sequenced.

Statistical and sequence analysis
Sequence alignments, translations, and comparisons were carried out using BIOEDIT (Version 7.0.9.0, Hall, T.A, 1999). The BLAST algorithm was used to search the NCBI GenBank (http://www.ncbi.nlm.hih.gov/) databases for homologous sequences.

Neighbor-joining trees (Saitou and Nei, 1987), were constructed on the basis of genetic distances, estimated by Kimura's (1980) two-parameter method, using MEGA 5 (Kumar *et al.,* 2001; http://www.megasoftware.net). The reliability of the trees was estimated by bootstrap confidence values (Felsenstein, 1985) and 500 bootstrap replications were used.

The NUC gene sequences used to construct the neighbour-joiningtree (Figure 3) (by NCBI Gen Bank accession numbers); the *S. aureus* NUC genes sequencesare CP011147, LNB31036, CP010944, CP010943, CP010940, CP010526, CP010300, CP010299, CP010298 and the *S. epidermidis* NUC gene sequences are HG813242 and CP000029.

RESULTS

Prevalence of Staphylococci:
The total prevalence of staphylococci from mastitic and apparently normal dairy cattle which isolated on baired –parker agar media are shown in table (1).

Coagulase test:
All staphylococci isolates were tested by slide coagulase test and tube coagulase test (table 2) to differentiate between coagulase positive and coagulase negative *S. aureus.*

PCR product:
PCR reaction revealed 21 positive amplicons of NUC gene and their length 267 bp in the presence of negative and positive controls which is characteristic for *S. aureus*

Predicted amino acid sequences:
The predicted amino acids of the NUC gene sequences detectedin this study are shown aligned with a reference predicted amino acid sequenceof the publishedgene bank sequences CP011147, LNB31036, CP010944, CP010943, CP010940, CP010526, CP010300, CP010299, CP010298.By estimating the coefficient variance of the *S. aureus* NUC gene sequences by using Wu-Kabat variability coefficient (Wu and Kabat, 1970), an amino acid site with a value of variability of 1 is monomorphic, while a site with a value exceeding 2 is polymorphic. The sequences isolated in this study show complete similarities between all local sequences and those sequences obtained from the gene bank except in one amino acidposition p58 (local sequence A15) which needs more investigation with large number of samples to detect if this variation is true variation or artefact.

Phylogenetic analysis:
The tree was constructed using the nucleotide sequences of 10 NUC genes isolated from S. aureus (local isolates, represented by boxes) and 9 sequences obtained from the genbank as mentioned before (represented by circle). The tree was rooted to 2 NUC genes of *S. epidermidis* sequences as out-groups (represented by triangle). All sequences were trimmed to similar length corresponding to the same region before generating the tree (figure 3). The tree showed high degree of similarities between all nuc sequences of local isolates or that imported from the gene bank.

Table 1: Staphylococci on baired-parker agar media

samples	NO.	Staphylococci (+ve)		Staphylococci (-ve)	
		NO.	%	NO.	%
Mastitic milk	77	43	55.8	34	44.2
Apparent normal milk	171	120	70.2	51	29.8
Total	248	163	65.7	85	34.3

Table 2: Detection of Coagulase positive and negative Staphylococci

No. of staphylococci	Test	CPS		CNS	
		NO	%	NO	%
163	Slide coagulase	24	14.7	139	85.3
	Tube coagulase	24	14.7	139	85.3

CPS: coagulase positive Staphylococci
CNS: coagulase negative Staphylococci

Table 3: *S. aureusconfirmation* by PCR test

No. of isolated *S. aureus*	Confirmed (+ve)		Confirmed (-ve)	
	No.	%	No.	%
24	21	87.5	3	12.5

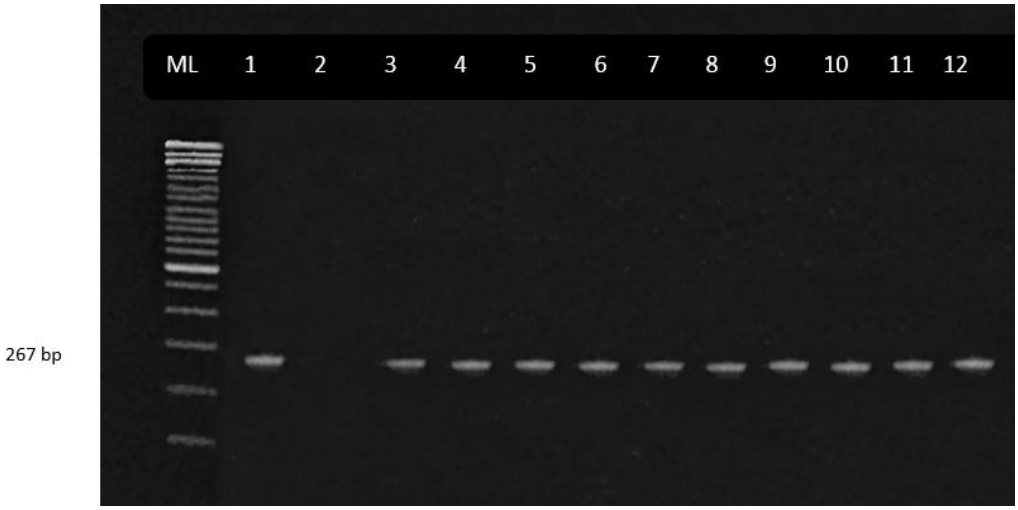

Figure 1: PCR products on gel electrophoresis (*nuc* gene) for characterization of *S. aureus*. Lane ML: 100 bp DNA marker, Lane 1: positive control for *S. aureusnuc* gene, Lane 2: negative control for *S. aureusnuc* gene and Lanes 3 to12: Positive *S. aureus* strains

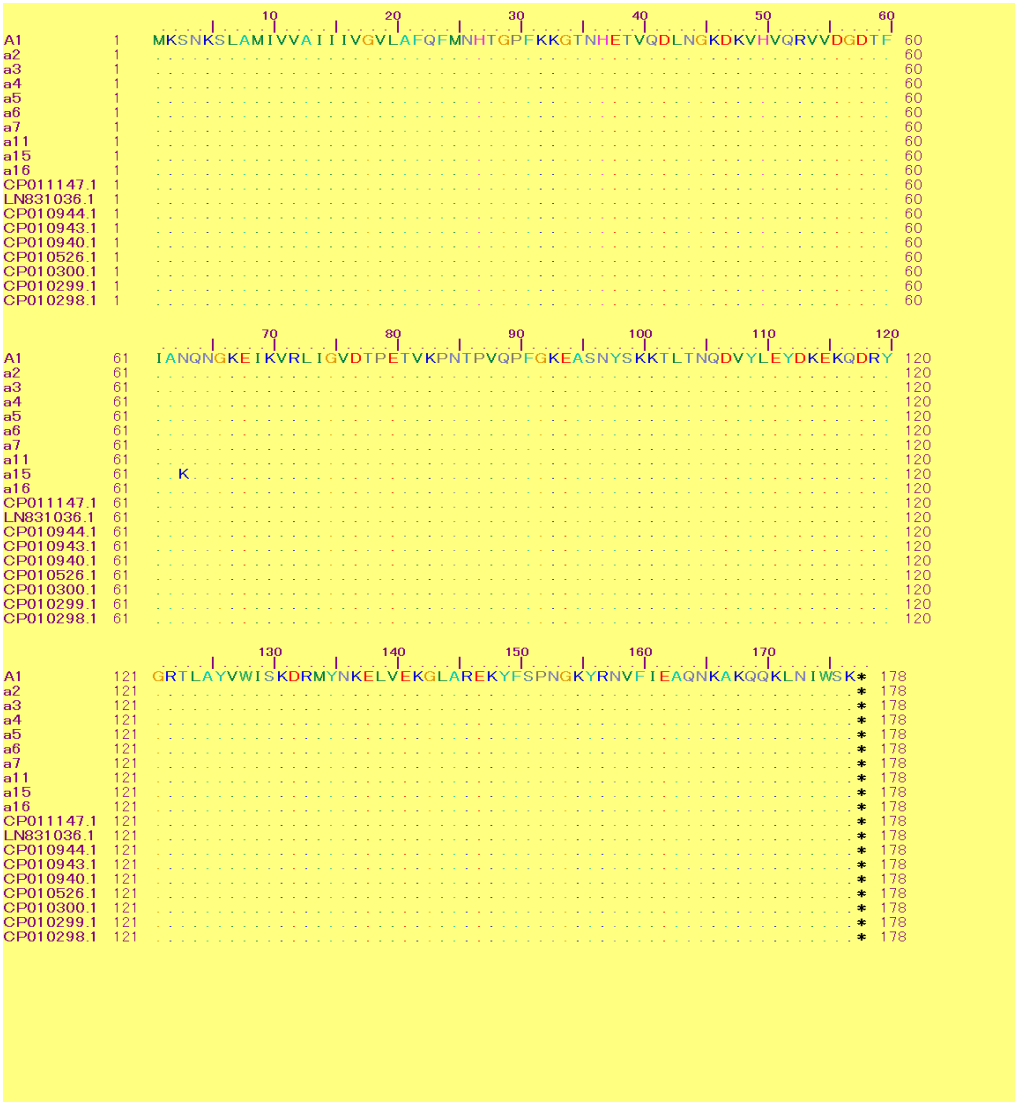

Figure 2: Alignment of the predicted amino acids of NUC gene, amino acids are presented in one letter-code. Dots represent residues identical to the AAs in the top sequence.

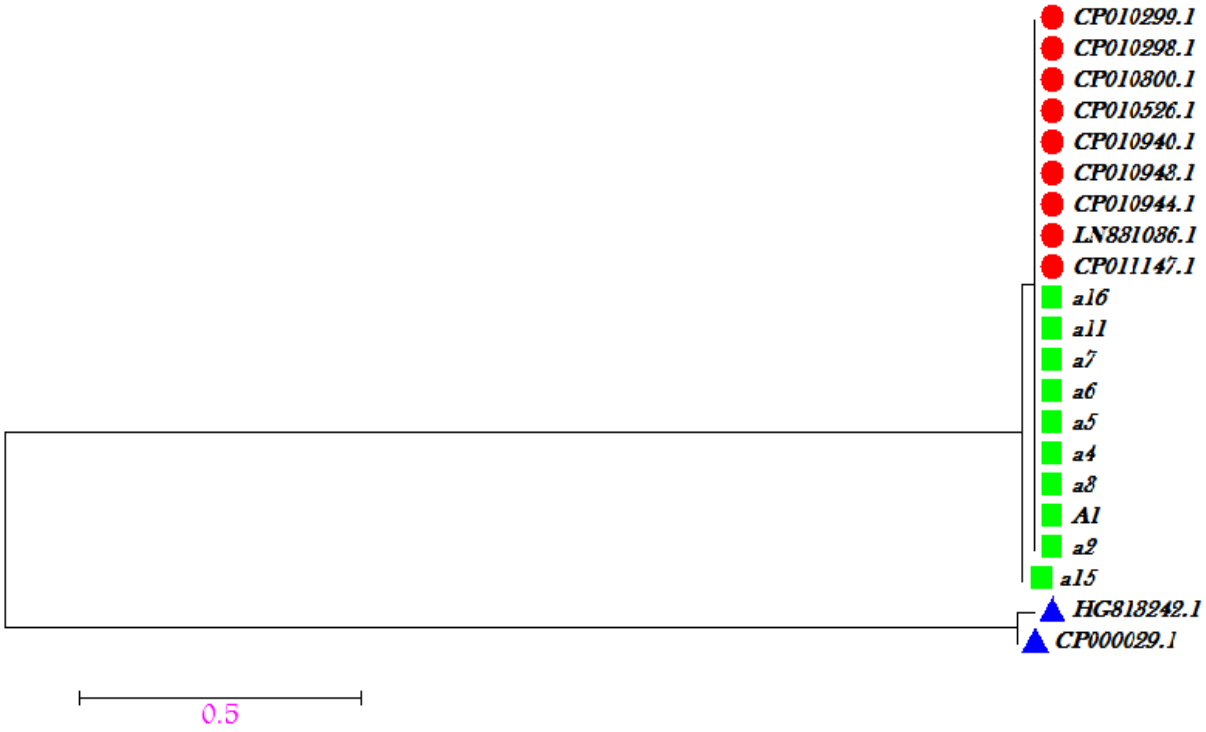

Figure 3: Phylogenetic analysis of Neighbour-joining tree of NUC gene sequences.

DISCUSSION

Detection of subclinical mastitis and the causative agent is the prerequisite for undertaking therapeutic measures. The PCR method has originally been used in human medicine, but currently the first attempts are reported at adopting it to diagnosis of mastitis in cattle (Phuektes *et al.,* 2003). Compared to conventional methods of identification of *S. aureus* isolates, the PCR method is less laborious, cheaper and more accurate. In the future it is likely to be 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.

Brakstad *et al.* (1992) have developed a PCR assay specific for *S. aureus* by targeting the nuc gene coding for staphylococcal thermonuclease. This assay was shown to be suitable for diagnostic purposes and this method was used in the present study for identification *S. aureus* isolates which gave nuc gene; amplicon size 276-bp that was successfully amplified from the genomic DNA of *S. aureus* (figure 1). 21 S. aureus isolates (87.5%) gave positive results by PCR in comparison to conventional coagulase test method (table 3).

The *nuc* genes annotated by homology comparison to thermonuclease were almost ubiquitous in the genus *Staphylococcus*. Current reports showed that regardless of their thermonuclease activity, all of the staphylococci investigated except for the *Staphylococcus sciuri* group carried *nuc* genes (Sasaki *et al.,* 2007), which is a species closely related to the genus *Staphylococcus*. Additionally, there were *nuc* genes in thermophilic bacteria, these results suggest that the *nuc* genes might have been derived from thermophilic bacteria and might have been acquired by the common ancestor of staphylococci after divergence from the *S. sciuri* group (Kwok and Chow., 2003).

Sequence alignments and phylogenetic analysis show a great level of similarities between different nuc gene alleles isolated from different isolates of *S. aureus* except a minor level of variations which needs further investigations (figure 2&3).

On conclusion, S. aureus is a highly significant pathogens inducing dramatic impact on the wide array of livestock production sectors which is reflected on the public health issue. The Nuc gene is used routinely for the characterization of *S. aureus* and the sequence analysis and phylogenetic studies revealed high degree of similarities which indicating this gene one of the monomorphic genes.

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دراسة تسلسل القواعد النيتروجينية وتوصيف النشوء والتطور لجين الثيرمونيكلاز في المكورات العنقودية الذهبية

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يعتبر التهاب الضرع من ابرز الأمراض المعدية التي تعيق صناعة الألبان في مصر وفي كل دول العالم وذلك للعديد من الأسباب ومن اهمها الطبيعة المزمنة والخسائر الاقتصادية التي يسببها هذا المرض وانتشاره على مدار العام كما يعد ميكروب المكورات العنقودية الذهبية احد اهم هذه الأسباب. في هذه الدراسة تم تجميع عدد ٢٤٨ عينة لبن من مزارع البان مختلفة منها ٧٧ عينة من حيوانات تعانى من التهاب الضرع السريري و ١٧١ عينة من حيوانات سليمة ظاهريا من المحتمل ان تكون مصابة ايضا ولكن بالتهاب الضرع التحت سريري وباستخدام اختبار التخثر وجد ان ٢٤ اعطت صورة ايجابية. هذه الدراسة استخدمت اختبار البلمرة المتسلسل الخاص باكتشاف جين الثيرمونيكلاز المميز لميكروب المكورات العنقودية الذهبية وكذلك تسلسل القواعد النيتروجينية وتوصيف النشوء والتطور لهذا الجين. اثبت تحليل تتابع القواعد النيتروجينية ودراسة النشوء والتطور لهذا الجين وجود درجة عالية من التشابه بين مختلف العزلات. وتعد هذه الطريقه الحديثة لدراسة الجينات اداة مثلى للوصول الى حالة فهم لمختلف السلالات الخاصة بميكروب المكورات العنقودية الذهبية المسببة لالتهاب الضرع في حيوانات المزرعة.