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MOLECULAR STUDY OF SOME VIRULENCE GENES DETERMINATE IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM MILK BOVINE MASTITIS.

Zeyad SH. Abbas*, Mohammad I. Nader and Ilham A. Khalaf

Genetic Engineering and Biotechnology Institute for Post Graduate Studies, Baghdad University, Iraq.

Al- Razi Center for Research and Diagnostic Kits, Ministry of Industry and Minerals, Baghdad, Iraq.

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***Corresponding Author**
Zeyad SH. Abbas
Genetic Engineering and Biotechnology Institute for Post Graduate Studies, Baghdad University, Iraq.

ABSTRACT

Mastitis is one of the common diseases of dairy cattle and an inflammatory response of the mammary glands tissue. *Staphylococcus aureus* is probably the most lethal agent *Staphylococcus aureus* is the main etiological agent of contagious clinical/subclinical mastitis in dairy herds. The aim of this study aims at the genotypic characterization of *S. aureus* strains isolated from dairy cows suffering from mastitis in the different regions (Abu Ghraib, Taji, Latifiya, Yousofia, Al-futailia) in Iraqi-Baghdad farms. A total of 60 *S. aureus* strains, obtained from 120 dairy cows, were phenotypically characterized and identified. Amplification of genes encoding clumping factor (*clfA*), coagulase (*coa*), thermonuclease (*nuc*), enterotoxin A (*entA*), and the gene segments encoding the immunoglobulin G binding region and the X region of protein A gene (*spa*) by Polymerase Chain Reaction was used for the genotypic characterization of isolated *S. aureus* strains. There was a statistical difference between isolates obtained from samples collected from cows infected in mastitis ($P < 0.01$). All of the isolates are posative for *nuc* and *spa* genes, 36 out of 60 isolates are posative of the polymorphic *ClfA* gene and X-region binding was detected in 55 isolates. Amplification of the *coa* gene yielded five different products in 4, 12, 23, 9 and 12 isolates. The amplification of the *entA* gene, was observed in 41 out of 60 isolates.

KEYWORDS: Bovine mastitis, *Staphylococcus aureus*, virulence factors, polymerase chain reaction (PCR), Iraq.

INTRODUCTION

Mastitis is one of the major causes of economic losses in dairy industry worldwide.[1,2,3] The major microorganism that coursed mastitis is *Staphyloccocus aureus* (*S. aureus*),[4] which responsible for intramammary infections in bovines, and is the main etiological agent of contagious clinical/subclinical mastitis in dairy herds.[5] *S. aureus* is an ubiquitous Gram-positive micro-organism commonly isolated from raw milk of dairy cattle suffering from mastitis. So its presence in raw milk is a major concern for the safety and the quality of traditionally dairy products.[6] It may cause a spectrum of pathologies in humans and animals.[7,8] There is considerable genetic heterogenecity through natural populations of *S. aureus*.[9,10] 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 .[11,12] *S. aureus* has a capacity to produce a large number of putative virulence factors.[13] 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.[14] However, at present nothing has been reported about the occurrence of these virulence factors among *S. aureus* isolates from Baghdad-Iraq, and about the possible distribution of single *S. aureus* clones as causativeagents of bovine mastitis at various farms.

MATERIALS AND METHODS

S. aureus strains were isolated from mastitis milk samples from 120 cows selected randomly from 5 farms at different localities of Baghdad, Iraq between October 2015 to January 2016. Isolation and identification of the strains were made by conventional methods,[15] by using API system.

Antibiotic susceptibility test

Susceptibility test was determined by standardized agar diffusion test [16] on Mueller-Hinton Agar (Difco) plates. Used antibacterial agents (Oxoid) were: Tigecycline (TGC, 15mg), Trimethoprim sulfa methoxazole (TS, 25mg), Amikacin (AK, 30 mcg), Ampicillin (Amp, 10 mcg), Trimethoprim (TM, 5 mg), Doxycycline (DXT, 30 mg), Gatifloxacin (GAT, 5 mg), Tetracycline (TE, 30 mg), chloramphenicol (C, 30 mcg), Cefoxitin (FOX, 30 mg), Netilmicin (NET, 30 mg), Azithromycin (ATH 15 mg) , Nitrofurantoin(300 mg). *S. aureus* ATCC 25923 was used as the control strain. Inhibition zones of growth were evaluated according to the NCCLS.

Processing of the samples for PCR assay: A volume of 1.5 ml of the post-enriched sample was centrifuged at 14,000g for 1 min, DNA was extracted using Presto Mini g DNA Bacteria Kit according to manufacturer's instructions (Geneaid, Korea). The extracted DNA was stored −20°C until use. The extracted DNA then quantified through measurement of its OD260 by ND‑2000 spectrophotometer (Thermo Scientific Inc., USA).

PCR amplification analysis: The virulence determinants investigated using the oligonucleotide primers included the genes *coa, clfA, spa, entA* and *nuc*. For all the genes, The polymerase chain reaction (PCR) amplification was performed in a final volume of 25µl containing 12.5 GoTaq®Green Master Mix (Promega) which contains (Taq plymerase, PCR buffer, MgCl2 and dNTPs), 200 ng of DNA template added 1µl of 10 pmol each primer and 9.5µl of nuclease free water, in the present study, the amplification parameters and primer sequence were used in (table1). The amplification of gene was carried out with Master cycler (Eppendrof, Germany). Amplified products were separated by agarose gel electrophoresis (2% agaros econtaining 0. 5 mg ethidium bromide in 0.5 × Tris - EDTA electrophoresis buffer) at 5V/cm for 2h and photographed under UV illumination.

Table 1. Primers and programs for amplification of the genes.

Gene	Sequence (5' - 3')	PCR program*	Size of amplified Products (bp)	References
Nuc	F: CGATTGATGGTGATACGGTT R: ACGCAAGCCTTGACGAACTAAAGC	1	27 9	[17]
Coa	F: ATAGAGATGCTGGTACAGG R: GCTTCCGATTGTTCGATGC	2	875, 800, 750 530,410.	[18]
ClfA	F: GGCTTCAGTGCTTGTAGG R: TTTTCAGGGTCAATA TAAGC	3	1042, 985.	[19]
EntA	F: AAAGTCCCGATCAATTTATGGCTA R: GTAATTAACCGAAGGTTCTGTAGA	4	216	[20]
Spa (IgG-binding)	F: CACCTGCTGCAAATGCTGCG R: GGCTTGTTGTTGTCTTCCTC	3	750, 920	[21]
spa (X-region)	F: CAAGCACCAAAAGAGGAA R: CACCAGGTTTAACGACAT	1	100, 250, 280, 300.	[22]
*1: 35 cycles 94°C-1min, 58°C-40 sec,72°C-1min ; 2: 30 cycles 94°C-60 sec61° C-40 sec,72°C-60 sec; 3: 30 cycles 94°C-60 sec, 62°C-60 sec,72°C60 sec; 4: 35 cycles 94°C-1 min59°C-1min, 72°C-60 sec. Initial denaturation at 95oC for 5 min and final extension at 72oC for 7 min.				

RESULTS AND DISCUSSIONS

After processing the samples, 60 (50%) *S. aureus* isolates were obtained as shown in (Table 2), farms had A and D 40% (10/25) isolates, Farms B and C had 57.69% (15/26) and 62.50% (15/24) isolates, respectively, Farms E had 50% (10/20) isolates. There was a statistical difference between isolates obtained from samples collected from cows infected in mastitis (P <0.01). According to cultural and biochemical properties, determined with the API Staph system, a

positive clumping factor reaction and a positive tube coagulase test all 44 isolates used in the present study could be identified as *S. aureus*. The antimicrobial susceptibility tests carried out in this study indicated the existence of susceptibility and resistance of *S. aureus* to some of the antimicrobials. The result of the present study shows that *S. aureus* isolates were resistant to Ampicillin (71.67%), Netilmicin (33.33%), Azithromycin (30%),Tetracycline (31.67%), Trimethoprim Sulfamethoxazole (3.33%), Gatifloxacin(26.67%), Doxycyline (23.33%), Cefoxitin (18.33%), Tigecycline (8.33%), Amikacin (8.33%), Chloramphenicol (8.33%), Nitrofurantion (6.67%) and Trimethoprim (5%) respectively, suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials. It is therefore, very important to implement a systemic application of an *in vitro* antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of intra-mammary infections.

Table 2. Percentages of *S. aureus* isolates from various farms of central Iraq.

Farm (No. of isolates)	***Nuc***	***ClfA***		***EntA***	***Coa***					***Spa IgG***		***Spa X***			
Mwt of genes	279	1042	985	216	875	800	750	530	410	920	750	300	280	250	100
Yousofia A (10)	10 (17%)	-	10 (17%)	9 (15%)	3 (5%)	-	2 (3%)	2 (3%)	3 (5%)	8 (13%)	2 (3%)	4 (7%)	1 (2%)	2 (3%)	3 (5%)
Abo-ghraib B (15)	15 (25%)	8 (13%)	-	7 (12%)	-	3 (5%)	-	5 (8%)	7 (12%)	12 (20%)	3 (5%)	5 (8%)	3 (5%)	-	7 (12%)
Al-futailia C (15)	15 (25%)	3 (5%)	7 (12%)	6 (10%)	-	4 (7%)	7 (12%)	2 (3%)	2 (3%)	14 (23%)	1 (2%)	4 (7%)	1 (2%)	4 (7%)	5 (8%)
Al-latifia D (10)	10 (17%)	3 (5%)	2 (3%)	9 (15%)	1 (2%)	4 (7%)	5 (8%)	-	-	10 (17%)	-	-	4 (7%)	3 (5%)	-
Taji E (10)	10 (17%)	3 (5%)	-	10 (17%)	-	1 (2%)	9 (15%)	-	-	10 (17%)	-	3 (5%)	5 (8%)	1 (2%)	-
Total	**60**	**17**	**19**	**41**	**4**	**12**	**23**	**9**	**12**	**54**	**6**	**16**	**14**	**10**	**15**

Amplification of *coa* gene yielded five different products of 875, 800, 750, 530 and 410 bp for 4, 12,23, 9 and 12 isolates respectively and gene polymorphism Figure (1). Amplification of the *clfA* gene yielded two different products of 1042 and 985 bp for 17 and 19 isolates respectively, and gene polymorphism Figure (2) . The amplification of the gene, segment encoding the IgG binding region of protein A (*spa*) revealed a size of 920 and 750 bp in 54 and 6 isolates respectively, and gene polymorphism was noted in isolates Figure (3) . The X-region binding of the *spa* gene produced an amplicon of 300, 280, 250, and 100 bp in 16, 14, 10 and 15 isolates, respectively Figure (4). The amplification of the enterotoxin A *entA* gene produced an amplicon of 216 bp in 41 out of 60 isolates Figure (5). The amplification of the extracellular thermonuclease *nuc* gene produced an amplicon of 279 bp in all isolates Figure (6).

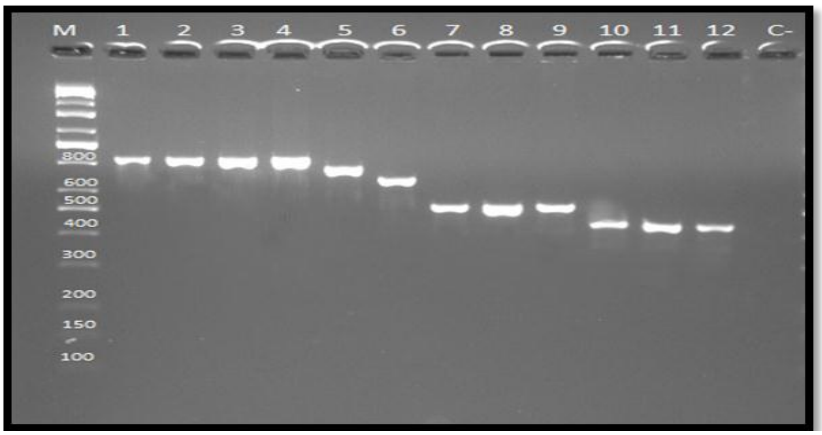

Figure (1): Agarose gel electrophoresis of PCR amplification products of *S .aureus*, *Coa* gene, line M:The DNA molecular wight marker (100-10000 bp), lines 1,2,3,4 (875 bp), 5 (800 pb), 6 (750 pb), 7,8,9 (530 pb), 10,11,12 (410 pb), line C- negative control.

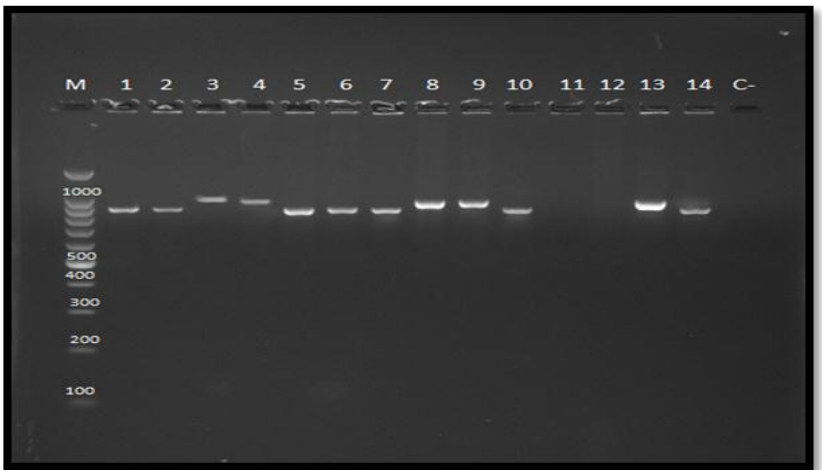

Figure (2): Agarose gel electrophoresis of PCR amplification products of *S. aureus*, *ClfA* gene, line M:The DNA molecular wight marker (100 bp), lines (3,4,8,9,13) positive amplification of (1042 bp), lines (1,2,5,6,7,10,14) positive amplification of (985 bp), lines (11,12) negative with the Clumping factor (*ClfA*) gene, line (C-) negative control.

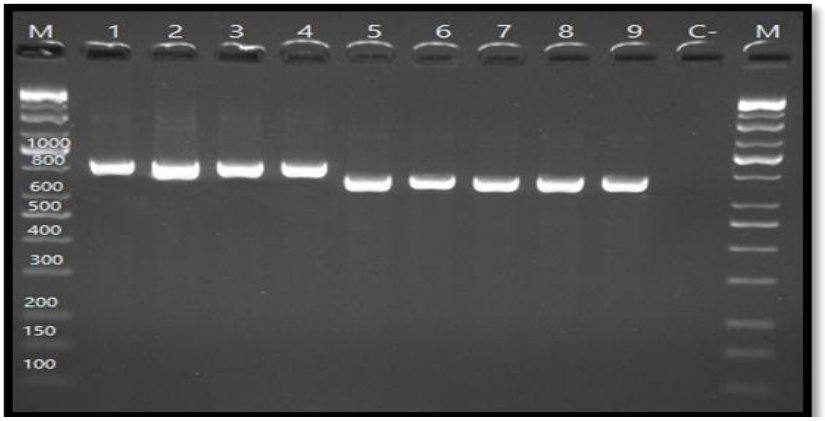

Figure (3): Agarose gel electrophoresis of PCR amplification products of *S.aureus*, *Spa* IgG binding region gene, line M:The DNA molecular wight marker (100-10000 bp), lines 1,2,3,4 (910 bp), 5,6,7,8,9 (750 pb),line C- negative control.

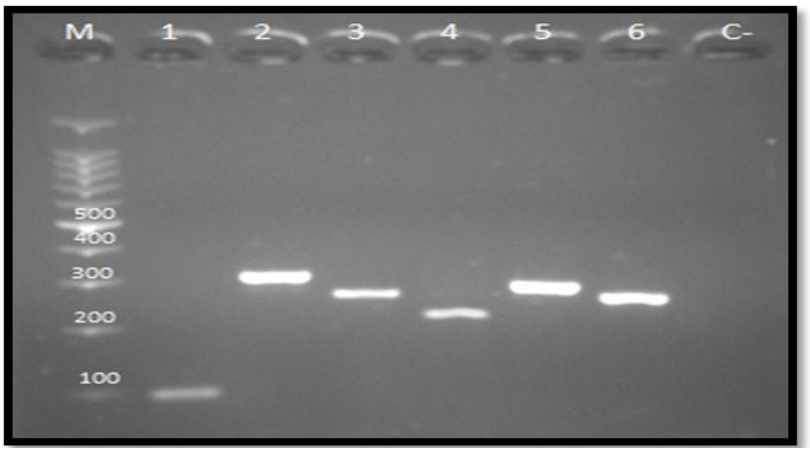

Figure (4): Agarose gel electrophoresis of PCR amplification products of *S.aureus*, *Spa* X-region gene, line M:The DNA molecular wight marker (100 bp), line 1 (100 bp), 2,5 (300 pb), 3,6 (280pb), 4 (250 pb), line C- negative control.

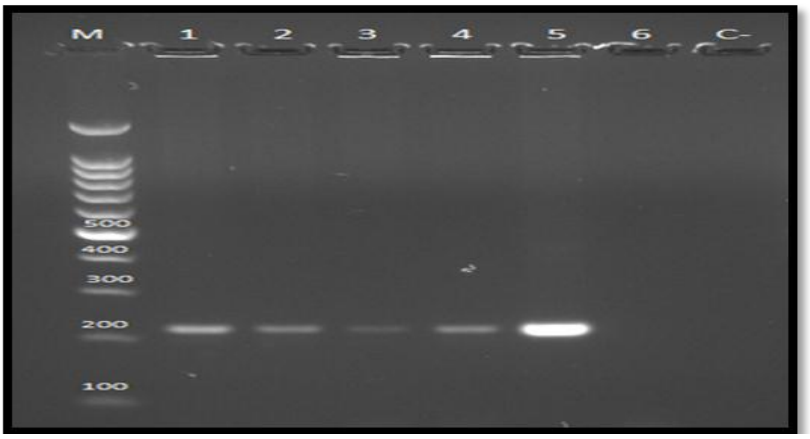

Figure (5): Agarose gel electrophoresis of PCR amplification products of *S.aureus*, *EntA* gene, line M:The DNA molecular wight marker (100 bp), lines (1-5) positive amplification of (216 bp) for *EntA* gene. Line (6) negative with the (*Ent A*) gene. line (C) negative control.

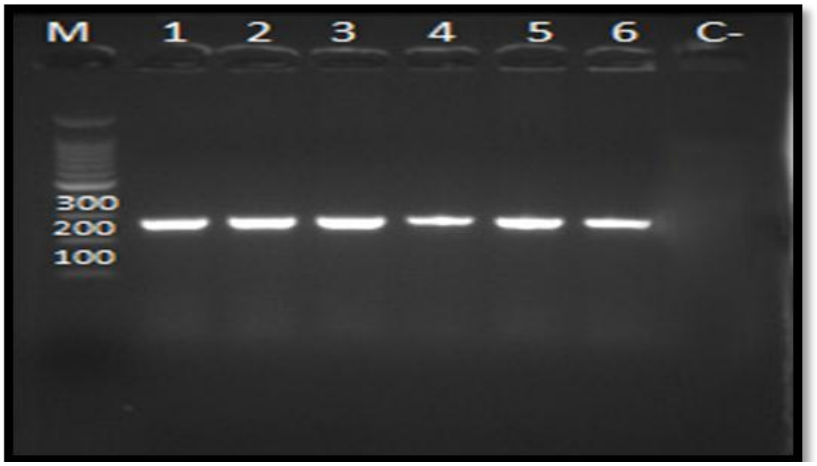

Figure (6): Agarose gel electrophoresis of PCR amplification products of *S.aureus*, *Nuc* gene, line M: The DNA molecular wight marker (100 bp), lines (1-6) positive amplification (279 bp) for *Nuc* gene. line (C-) negative control.

S. aureus has been recognized as a pathogen in human and animal infections. In the present study, Sixty *S. aureus* strain isolated from clinical bovine mastitis cases were identified and further characterized by PCR amplification of various virulence genes encoding clumping factor (*clfA* gene), coagulase activity *(coa* gene), gene segments encoding the immunoglobulin G-binding region and X-region of protein A *(spa*) and stable thermonuclease (*nuc* gene) activity.

The *coa* , *clfA* and *spa* (IgG-binding region and X-region) genes investigated in the present work exhibited typical gene polymorphism, Comparable PCR based detection studies of the virulence genes have been described by other investigators.[23,12]

The *spa* gene segments encoding the X-repetitive region are known to consist of a variable number of small repeats.[24] It is thought that the spa domain encoding the X-region may serve to extend the N-terminal IgG-binding portion of the protein through the cell wall. It was interesting to note that isolates from the same farm exhibited polymorphism among the *coa, clfA* and *spa* genes. The ability of *S. aureus* to adhere to extracellular matrix proteins is thought to be essential for the colonization and the establishment of infections.[24]

PCR analysis of the other virulence genes revealed the *nuc* gene in 60, of the 60 strains investigated, suggesting an important role of these elements in the pathogenecity of bovine mastitis. As well, *ent A* gene was present most the strains (41 strains). In contrast, not occurrence of *entA* genes by other investigators.[23,25]

In the present study, *S. aureus* isolates from bovine mastitis to differ in their gene patterns Phenotypic and genotypic characterization might provide a better understanding of the distribution of the prevalent *S.aureus* clones among bovine mastitis isolates. This can aid in the investigation and control of *S. aureus* infections in dairy herds.

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