Molecular detection of some virulence genes of *S. aureus* isolated from mastitic Cows by PCR.

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A B S T R A C T

In this study a total of 1200 quarter milk samples were selected from 300 cows at different localities of El-Kaliobia Governorate were examined bacteriologically and revealed that 57 *S.aureus* isolated from both clinical and subclinical mastitis with incidence of 45.6% of total isolates. Molecular typing of *S. aureus* by PCR revealed that all isolates (100%) (12 random *S.aureus* isolates) were positive for the *16 SrRNA* genes of *S. aureus*. Genotyping of virulence genes encoding factors of *S. aureus* revealed that two isolates were positive for (*tst-1)* gene (16.7%), three isolates positive for (*etb*) 25%, nine isolates positive for each of (*icaD* and *femA*) genes (75%), seven isolates positive for (*hlg)* (58.3%), eight isolates positive for (*nuc*) (66.7%).

Keywords: Mastitis, *S. aureus*, PCR, virulence genes.

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1. INTRODUCTION

Mastitis is the most prevalent infectious disease affecting dairy cattle which have many adverse economic implications worldwide represented by decrease in quantity and quality of milk components and shorten the reproductive life of affected animals. Due to limitation of cultural and biochemical methods for isolation of bacteria, new methods using (PCR) based on the amplification of DNA coding for 16S *r RNA* and 23 *S r RNA* region have been successfully applied for identification of bovine mastitis pathogens (Riffon et al., 2001). This method identification bacterial pathogens in hours rather than days required by conventional cultural method. Also it is specific, sensitive, and cheap can discriminate between closely related organisms. The major advantage of PCR lies in the possibility of using only nano grams of nucleic acid samples. So presence of pathogens could be detected at the earlier stages of infection and in carriers when the number of bacteria in milk may be very low (Riffon et al., 2001). The pathogenicity of *S. aureus* could be attributed to the virulence factors the bacteria produce. These virulence factors include , adhesion factors (collagen - binding protein, fibronectin-binding protein A/B, Clumping factors A(*clfA)*, a fibrinogen binding surface protein of *S. aureus*, is an important virulence factor in septic arthritis and intracellular adhesion *(icaA)* , toxins (Enterotoxins, Toxic shock syndrome toxin-1, Panton-Valentine, Leukocidin, Hemolysins, Coagulase that clot blood, protease and protein A .The aim of this work was based on application of (PCR) as a rapid and sensitive test for direct detection of *S.aureus* causing mastitis using species specific primers and its evaluation in relation to bacteriological examination ,more over genotyping of isolated *S.aureus* through detection of some virulence factors

encoding genes(*etb*, *icaD, tst-1, femA, nuc, hlg)*.

2. MATERIAL AND METHODS

2.1. Milk collection

A total number of 1200 individual quarter milk samples according to National Mastitis Council (1990) were collected from 300 Cows from different localities at El-Kaluobia Governorate. From which 1060 quarter milk samples were collected from 265 Cows with apparently normal udders & milk secretions while the remaining 140 quarter milk samples were collected from 35 Cows showed clinical mastitis (by using CMT).

2.2. Bacteriological examination

A loopful of each quarter milk sample was streaked onto nutrient agar medium, Mannitol salt agar, blood agar and Baired parker agar. All plates were incubated for 24 hours at 37^0c. The developed colonies were picked up sub culturing for purification of the isolates. The purified colonies were subjected for morphological identification by Gram's stain &biochemical tests (Arora, 2003).

2.3. Direct detection of S.aureus using conventional PCR assay

Extraction of *DNA* was performed according to QIAamp DNA mini kit instructions QIAGEN (USA). Preparation of uniplex PCR Master Mix for each of the tested genes was performed according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit (Emerald Amp GT PCR master mix (2X pre-mix) and PCR grade water (Dnase free water). Used for the PCR as in tables(2 and 3). The Oligonucleotide were designed against *16 Sr RNA* gene as follow the forward primer CCTATAAGACTGGGATAACTTCGGG and the reverse primer CTTTG-AGTTTCAACCTTGCGGTCG (Mason *et al.,* 2001).

2.4. Genotyping of virulence gene encoding factors by cPCR

Seven pairs of primers were supplied from metabion (Germany) or Biobasic (Canada). They have specific sequence and amplify specific products as shown in Table (4).

3. RESULTS

3.1. Incidence of bacteriological examination

Bacteriological examination of (35) clinically mastitic quarter milk samples and (76) subclinically mastitic quarter milk samples revealed that the predominant bacterial species isolated from the examined quarter milk samples was *S.aureus* 57 isolates with incidence of (45.6%) as showed in table (1).

3.2. Molecular identification of S.aureus

A primer pairs for the gene (16*SrRNA*) were designed for all the tested *S.aureus* isolates (random 12 isolates of *S.aureus*). The amplicon size of the examined gene (16SrRNA) at 791 bp and the results showed that all of the tested *S.aureus* strains were positive for the 16*SrRNA* gene (100%).

3.3. Genotyping of virulence gene encoding factors by cPCR

Detection of exfoliative B gene(etb), Fembria A toxin gene *(femA), γ* hemolysin gene (*hlg*), intra cellular adhesive toxin D (*icaD*), thermo-nuclease gene (*nuc*), toxic shock syndrome toxin-1 (*tst-1*). Agarose gel photo–documentation on PCR products showed the amplicon size of the examined gene (*etb*) at 226 bp. The result showed that the gene encoding the exfoliative B gene (*etb*) was found among 25% of the *S.aureus* isolates as showed in table (5). Agarose gel photo-documentation on PCR products showed the amplicon size of the examined gene (*fem A*) at 132 bp. The result showed that the gene encoding the Fembria A. (*femA*) was found among 75% of the *S.aureus* isolates as showed in table (5). Agarose gel photo-documentation on PCR products showed the amplicon size of the examined gene (*hlg*) at 937 bp. The result

Table (1) Bacterial species isolated from positive milk samples of both clinical and Subclinical mastitic Cows

Bacterial isolates	Clinical mastitis (35)		Positive CMT (76)		Total (111)	
	No.	%	No.	%	No.	%
Total *Staphylococci*:	24	55.8	40	48.8	64	51.2
Coag. Post. *S. aureus*	21	48.8	36	43.9	57	45.6
Coag. Neg. *S. epidermidis*	3	7.0	4	4.9	7	5.6
Total *Streptococci*:	10	23.3	19	23.2	29	23.2
S. agalactiae	3	7.0	10	12.2	13	10.4
S. dysgalactia	4	9.3	5	6.1	9	7.2
S. uberis	3	7.0	4	4.9	7	5.6
E. coli	8	18.6	20	24.4	28	22.4
Klebsiella spp.	1	2.3	2	2.4	3	2.4
Ps. aeruginosa	0	0.0	1	1.2	1	0.8
Total	43	100.0	82	100.0	125	100.0

Table (2) PCR components used in the PCR technique

Component	Volume / reaction
Master mix(Emerald Amp GT PCR 2x premix)	12.5 µl
Forward(F) primer (20 pmol)	1 µl
Reverse (R)primer(20pmol)	1 µl
PCR grade Deionized H2O	5.5 µl
DNA template	5 µl
Total volume	25 µl

Table (3) Cycling conditions of the primers during cPCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
etb	94˚C	94˚C	50˚C	72˚C	35	72˚C
	5 min.	30 sec.	30 sec.	30 sec.		7 min.
Tst-1	94˚C	94˚C	50˚C	72˚C	35	72˚C
	10 min.	45 sec.	45 sec.	45 sec.		10 min.
16SrRNA	94˚C	94˚C	55˚C	72˚C	35	72˚C
	5 min.	1 min.	1 min.	1 min.		10 min.
hlg	94˚C	94˚C	55˚C	72˚C	35	72˚C
	10 min.	1 min.	1 min.	1 min.		10 min.
icaD	94˚C	94˚C	49˚C	72˚C	35	72˚C
	10 min.	45 sec.	45 sec.	45 sec.		10 min.
femA	94˚C	94˚C	50˚C	72˚C	35	72˚C
	5 min.	30 sec.	30 sec.	30 sec.		7 min.
nuc	94˚C	94˚C	55˚C	72˚C	35	72˚C
	10 min.	45 sec.	45 sec.	45 sec.		10 min.

Table (4) Oligonucleotide primers sequences

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
Etb	F/ ACAAGCAAAAGAATACAGCG R/GTTTTTGGCTGCTTCTCTTG	226 bp	Mehrotra et al., 2000
tst	F /ACCCCTGTTCCCTTATCATC R /TTTTCAGTATTTGTAACGCC	326 bp	
femA	F /AAAAAAGCACATAACAAGCG R /GATAAAGAAGAAACCAGCAG	132 bp	
16SrRNA	F /CCTATAAGACTGGGATAACTTCGGG R / CTTTGAGTTTCAACCTTGCGGTCG	791 bp	Mason et al., 2001
hlg	F/GCCAATCCGTTATTAGAAAATGC R/CCATAGACGTAGCAACGGAT	937 bp	Kumar et al., 2009
icaD	F /AAA CGT AAG AGA GGT GG R /GGC AAT ATG ATC AAG ATA	381 bp	Ciftciet al., 2009
nuc	F /ATATGTATGGCAATCGTTTCAAT R /GTAAATGCACTTGCTTCAGGAC	395 bp	Gao et al., 2011

Table (5) Results of the virulence factors encoding genes

Name of gene	No. of isolates	percentage
Toxic shock syndrome toxin-1(*tst-1*)	2	16.7 %
Exfoliative toxin B(*etb*)	3	25.0 %
Intra cellular adhesive toxin D(*icaD*)	9	75.0 %
Gamma Hemolysin toxin (*hlg*).	7	58.3 %
Thermo-nuclease toxin(*nuc*)	8	66.7 %
Fembria A toxin(*femA*)	9	75.0 %

showed that the gene encoding the gamma hemolysin (hlg) was found among 58.3% of the S.aureus isolates with no evidence of gene polymorphism as showed in table (5). Agarose gel photo – documentation on PCR products showed the amplicon size of the examined gene (icaD) at 381 bp. The result showed that the gene encoding the intra cellular adhesive D. (IcaD) was found among 75% of the S.aureus isolates with no evidence of gene polymorphism as showed in table (5). Agarose gel photo – documentation on PCR products showed the amplicon size of the examined gene (nuc) at 395 bp. The result showed that the gene encoding the thermo-nuclease. (nuc) was found among 66.7 % of the S.aureus isolates with no evidence of gene polymorphism as showed in table (5). Agarose gel photo–documentation on PCR products showed the amplicon size of the examined gene (tst-1) at 326 bp. The result showed that the gene encoding the toxic shock syndrome toxin 1. (tst-1) was found among 16.7% of the S.aureus isolates with no evidence of gene polymorphism as showed in table (5).

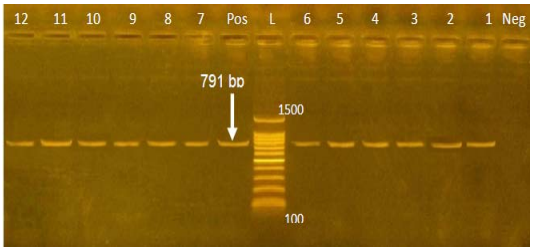

Photo (1) Agarose gel photo documentation of conventional PCR on genetic material extracted from S.aureus strains as a molecular typing for detection of 16Sr RNA gene. Lane L: molecular weight marker (100 – 1500 BP). Lanes 1-12: positive samples S.aureus16Sr RNA gene with amplicon size of 791 bp. Lane Neg: negative control. Lane Pos.: positive control

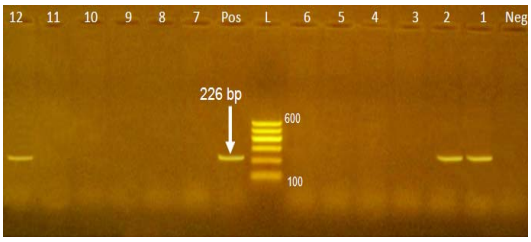

Photo (2) Agarose gel photo documentation for detection of virulence factor encoding gene (etb) of S.aureus as a genotyping identification of the isolates. Lane L: molecular weight marker (100 – 600 bp). Lanes 1, 2& 12: positive samples for (etb) gene with amplicon size of 226 bp. Lanes 3,4,5,6,7,8,9,10 & 11 : negative samples for the gene (etb) gene. Lane Neg: negative control. Lane Pos.: positive control

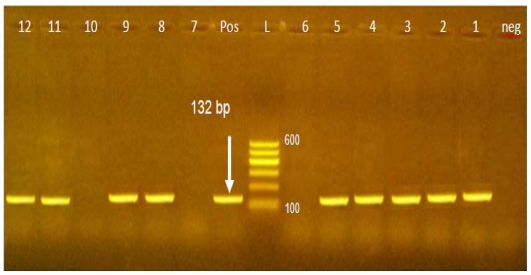

Photo (3) Agarose gel photo documentation for detection of virulence factor encoding gene (femA) of *S.aureus* as a genotyping identification of the isolates. Lane L: molecular weight marker (100 – 600 bp). Lanes 1,2,3,4,5,8,9,11&12: positive samples for (*fem A*) gene with amplicon size 132 bp. Lanes 6, 7& 10: negative samples for the gene (*fem A*) gene. Lane Neg: negative control. Lane Pos.: Positive control

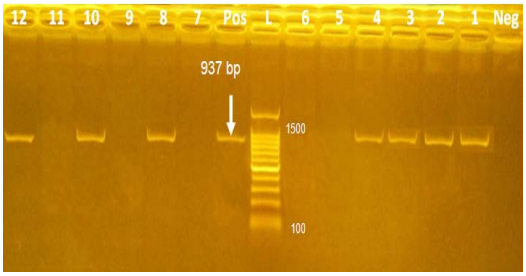

Photo (4) Agarose gel photo documentation for detection of virulence factor encoding gene (*hlg*) of *S.aureus* as a genotyping identification of the isolates. Lane L: molecular weight marker (100 – 600 bp). Lanes 1, 2, 3, 4, 8, 10&12: positive samples for (*hlg*) gene with amplicon size of 937 bp. Lanes 5, 6, 7, 9 & 11: negative samples for the gene (*hlg*) gene. Lane Neg: negative control. Lane Pos.: positive control

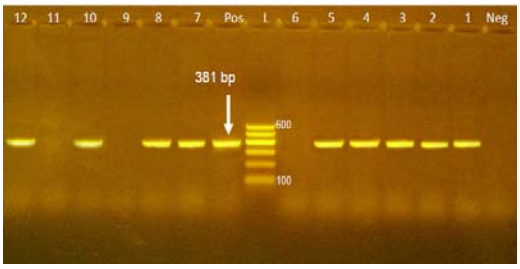

Photo (5) Agarose gel photo documentation for detection of virulence factor encoding gene (*icaD*) of *S.aureus* as a genotyping identification of the isolates.Lane L: molecular weight marker (100 – 600 bp). Lanes 1,2,3,4,5,7 ,8,10 & 12: positive samples for (*icaD*) gene with amplicon size of 381 bp . Lanes 6, 9 & 11: negative samples for the gene (*icaD*) gene.

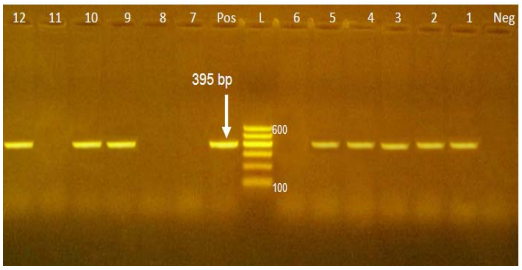

Photo (6) Agarose gel photo documentation for detection of virulence factor encoding gene (nuc) of *S.aureus* as a genotyping identification of the isolates. Lane L: molecular weight marker (100 – 600 bp). Lanes 1,2,3, 4,5,9,10 & 12: positive samples for (*nuc*) gene with amplicon size of 395 bp . Lanes 6, 7, 8, & 11: negative samples for the gene (*nuc*) genel

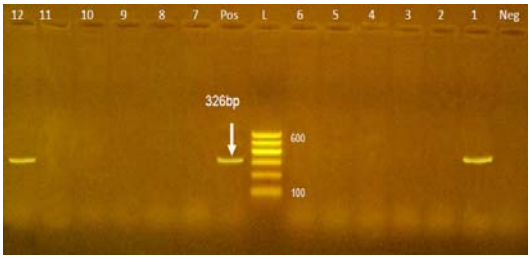

Photo (7) Agarose gel photo documentation for detection of virulence factor encoding gene (*tst-1*) of *S.aureus* as a genotyping identification of the isolates. Lane L: molecular weight marker (100 – 600 bp). Lanes 1 & 12**:** positive samples for (*tst-1)* gene with amplicon size of 326 bp. Lanes 2, 3, 4, 5,6,7,8, 9, 10 & 11negative samples for the gene (*tst-1*). Lane Neg**:** negative control. Lane Pos.: positive control.

4. DISCUSSION

The results of bacteriological examination of 178 mastitic milk samples (38 clinical mastitic ,140 subclinically ones)(table 1)showed that 35(92.1%) of clinically mastitic milk samples were positive with 27 (77.1%) pure single cultures and eight (21.1%) mixed ones .While76 subclinical mastitic milk samples (54.3%) were positive with 69 (49.3%) pure single cultures and seven (5%) mixed ones .Similar results were obtained by Karimuribo et al., 2008; and Ibrahim 2007 .Concerning PCR technique it is a rapid, sensitive and specific method for staphylococcal spp. differentiation at both strain or subspecies level in about 6-8 hours of testing and can detect staphylococcal from milk samples of antibiotics treated animals. Using *S.aureus* specific primers that encoding *16 SrRNA* genes for testing 12 random *S.aureus* samples which were positive for CMT& bacteriological examination. The obtained results showed that all *S.aureus* isolates amplified at 791 bp. These results were come in accordance with those recorded by Riffon et al., (2001), El-Sayed. (2010) and Suleiman et al., (2012) who found a correlation of 100% between PCR and QMS culture method. The high virulence of *S.aureus* is mostly due to its ability to produce a large number of virulence factors that can contribute to different ways to their pathogenicity Momtaz et al., (2011) namely coagulase gene (*coa*), thermo nuclease gene (*nuc*) & the gene segment encoding the immunoglobulin G region (*spa –IgG*). Also *S.aureus* expresses surface – associated anti – opsonic proteins (protein A, clumping factor A) an extracellular poly saccharides layer & a capsule that impairs phagocytosis Buzzola et al 2007 moreover *S.aureus* has the ability to secrete enterotoxins which play an important role in the developing of the disease as suggested to be involved in the epithelium damage of the mammary gland even minor trauma. Concerning the *nuc* gene and as shown in (photo 6) the observed result was 66.7% which nearly similar to that obtained by Mounir (2010) who found that the *nuc* gene expressed as (78 %). while it differenced with that of Memon et al., (2013) and Soliman (2014) who detected that the (*nuc* gene) in 85 % and 98.9 % of samples respectively.

Also these results were not agreed with that reported by El-Sayed et al (2006), who found that all *S.aureus* isolates were +ve for *nuc* gene (100 %). Concerning (*etb* gene) as shown in (photo 2) the result was 25 %, this result was contradicted for that obtained by El-Sayed et al., (2006) who found that all tested strains were negative for (*etb* gene). Similar results recorded by Cremonesi et al., (2013) and Memon et al., (2013). Concerning (*tst-1*gene) the result was 16.67%. this result was nearly similar to previous result of Lim SukKyung et al., 2004 who determined that (tsst-*1* gene) in 23.9 % of tested strains. This result was higher to that of Oliveira et al., (2011) who found that *tsst-1* gene was in 8.4 % of tested isolates. Also these result were not agreed with that recorded by El-Sayed et al., (2006) who found that all tested strains were negative for (*tsst-1* gene) and Stephan et al., 2001 who found that 67.7 % of isolates were positive for (*tsst-1*) gene. Concerning the (*femA*) gene the result was 75 %, this result was nearly parallel to that reported by Kobayashi et al., (1994) who found that 89.4% of total isolates harbor the *femA*

gene. The results of PCR for amplification of gamma hemolysin toxin (*hlg*) gene in *S.aureus* isolates (photo, 4) showed that, the *hlg* gene was amplified in 7 (58.3%) strains giving product of 937 bp. Similar findings were recorded by Memon et al., (2013) . The results of PCR for amplification of intra cellular adhesive toxin D (*icaD*) gene in *S.aureus* isolates (photo 5) showed that the *icaD* gene was amplified in 9 (75.0%) strains giving product of 381 bp. Similar findings were recorded by Memon et al., (2013).

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