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RESEARCH ARTICLE

Association between *agr* Alleles and Toxin Gene Profiles of *S. aureus* Isolates from Human and Animal Sources in Egypt

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Abstract

Staphylococcus aureus is a persistent human and animal pathogen responsible for a large spectrum of infections due to the production of secreted and cell surface-associated proteins under regulation of the *agr* (accessory gene regulator) locus. This study was undertaken to find out the prevalence of *Staphylococcus* species in Sharkia province, Egypt in 2012 and to investigate the potential association between *agr* groups and the occurrence of toxin genes among *S. aureus* isolates. In the present study, 46 *Staphylococcus* species were isolated from 141 different samples (32.62%), 26 were identified as *S. aureus* and 20 were classified as coagulase negative staphylococci (CoNS). In particular, 15 *S. aureus* strains identified phenotypically were confirmed genotypically by polymerase chain reaction (PCR) amplification of thermonuclease (*nuc*) and coagulase (*coa*) genes. All of them possessed *nuc* gene and were grouped into 15 different groups according to *coa* gene polymorphism. Moreover, typing of the *agr* operon revealed the predominance of *agr* group I (93.33%). Interestingly, all 15 *S. aureus* isolates tested were toxin producers with a high prevalence of staphylococcal enterotoxin A (*sea*) gene (86.67%). None of the isolates harbored the genes encoding *seb*, *sec*, *sed* and exfoliative toxin B (*etb)*. All *sea* and *tst* (toxic shock syndrome toxin) positive strains were of *agr* type I. Overall, toxin and coagulase genotyping could be more discriminatory than *agr* typing of the isolates. Finally, we can speculate that the preferential association between certain *agr* alleles and certain toxin genes may make the activation of virulence factors more efficient.

Introduction

S. aureus is an extremely versatile human pathogen responsible for a broad range of nosocomial and community-acquired infections due to an impressive array of virulence determinants (Plata et al., 2009). *S. aureus* is also responsible for intramammary infections in bovines and is the main etiological agent of contagious clinical/subclinical mastitis in dairy herds (Gilbert et al., 2006; Taverna et al., 2007). The pathogenicity of this organism is a complex process involving a diverse array of virulence factors that are coordinately expressed during different stages of infection by a network of virulence regulators (Cotar et al., 2010). These virulence factors include a variety of exoproteins and cell wall-associated proteins (Arvidson and Tegmark, 2001). Nearly all *S. aureus* strains secrete a group of exoproteins such as exotoxins and enzymes, including coagulase, nucleases, proteases, lipases, hyaluronidase and collagenase. Staphylocoagulase (SC) causes coagulation of plasma and is regarded as a marker for discriminating *S. aureus* from other less pathogenetic staphylococci called coagulase-negative staphylococci (Kloos and Schleifer, 1986). The nuclease gene was also used to identify *S. aureus* (Rushdy et al., 2007). It encodes the TNase production which has species-specific sequences and amplification of the *nuc* gene has a potential for the rapid diagnosis of *S. aureus* infections (Brakstad et al., 1992; Costa et al., 2005). However, depending on the strain, some *S. aureus* can produce exotoxins such as toxic shock syndrome toxin (TSST-1) and exfoliative toxins (ETs)

and some strains express heat stable staphylococcal enterotoxins (SEs) (Becker et al., 2003). As major virulence factors in *S. aureus*, TSST-1, ETs (A and B) and SEs are pyrogenic toxins that have been implicated in host colonization, invasion of damaged skin and mucus, gastrointestinal infection and evasion of host defense mechanisms. They are responsible for specific acute staphylococcal toxaemia syndromes (Adwan et al., 2006; Udo et al., 2009). TSST-1 producing *S. aureus* can cause multiorgan disease called toxic shock syndrome (TSS). It is encoded by the *tst* gene which is located on the bacterial chromosome within 15 to 19 kb genetic elements designated *S. aureus* pathogenicity islands (SaPIs) (Ruzin et al., 2001). The two ETs (ETA and ETB), in conjunction or independently, are implicated in the cause of staphylococcal scalded skin syndrome (SSSS) (Iandolo, 1989). Enterotoxigenic *Staphylococcus aureus* is one of the major pathogens causing food poisoning cases worldwide (Dinges et al., 2000). It is documented that more than 70% of *S. aureus* strains produced one or more enterotoxins (Balaban and Rasooly, 2000). Eighteen different types of enterotoxins such as SEA-SEE, SEG-SER and SEU encoded by *sea- see*, *seg-ser* and *seu* genes, respectively have been reported (Ferry et al., 2005). The coordinated expression of *S. aureus* secreted and cell wall-associated virulence factors is regulated by a complex network including the quorum-sensing (QS) system *agr* and the well characterized virulence gene regulators (Cotar et al., 2010). The *agr* locus consists of two divergent transcription units RNAII and RNAIII driven by two promoters, P2 and P3, respectively. The P2 operon encodes a two component signaling module, of which AgrC is the membrane sensor and AgrA is the response regulator. It also encodes two proteins, AgrB and AgrD, which combine to produce and secrete an autoinducing peptide (AIP) that is the ligand for AgrC. The AIP interacts with AgrC in the membrane to activate AgrA, which upregulates transcription both from its own promoter P2, amplifying the response and from the *agr* P3, initiating the production of a 0.5-kb highly conserved *agr* transcript, RNA III (the intracellular effector of target gene regulation), which in turn upregulates certain extracellular toxins and enzymes expressed post exponentially (toxins, hemolysins, proteases, nucleases and lipases) and repressing some exponential-phase surface proteins such as protein A, coagulase and probably fibronectin binding protein under conditions of high autoinducer concentration as high bacterial cell densities (Lindberg et al., 1990; Novick, 1999; Novick et al., 1993). *S. aureus agr* is a 3-kb locus showing highly conserved and hypervariable regions. Sequence diversity in the variable region, comprising the last one-third of AgrB, AgrD and the first half of AgrC, has generated the 4 *agr* specificity groups in *S. aureus* (I – IV) and it is the target of PCR amplification for defining *agr* types (Jarraud et al., 2002; Novick, 2003). Therefore, it is important to determine *agr* genotypes and toxin gene profiles of *S. aureus* recovered from different human and animal sources in Egypt using multiplex PCRs to understand the genetic and pathogenic relatedness, as well as the epidemiology of *S. aureus*. Finally, it would be interesting to establish a possible relationship between the *agr* groups and the occurrence of toxin genes to investigate the contribution of the *agr* operon to the virulence of *S. aureus* strains.

2. Materials and methods

2.1 Clinical specimens

One hundred and forty one different samples were collected from animal (96) and human sources (45) from different areas in Sharkia province, Egypt in 2012. The samples from animal origin included mastitic cow milk (64) and food specimens (32) [sausage (7), burger (11) and minced meat (14)], while those from human subjects were urine (8), pus (7), sputum (6), CSF (6), blood (7), pericardial fluid (5) and peritoneal fluid (6). They were transported in an ice box and microbiological examination was performed within 24 h.

2.2 Phenotypic characterization of staphylococcal isolates

Primary isolation of staphylococcal isolates was carried out onto mannitol salt agar (Oxoid). Single, well-isolated colony from overnight cultures was subcultured onto blood and milk agar for testing the beta hemolysis (Skalka et al., 1979) and pigment production. Identification of the isolates was realized on the base of standard bacteriological methods including cultural characteristics, Gram's stain and biochemical tests such as O/F (oxidative/fermentative), catalase and tube coagulase tests (Bannerman, 2003). Furthermore, *Staphylococcus* species were biotyped using the API 20 Staph identification kit (BioMerieux, Marcy l'Etoile, France). All isolates were stored with 50% glycerol at –70°C until required.

2.3 Genomic DNA extraction

Total DNA was extracted from *S. aureus* grown on mannitol salt agar plates by using the boiling approach as described previously (Zhang et al., 2004). In brief, for rapid DNA extraction, one to five colonies of each freshly subcultured strain were suspended in 50 µl sterile distilled water and heated at 99°C for 10 min. After centrifugation at 30,000 xg for 1 min, the supernatant was used as a DNA template and stored at -20ºC until PCR was performed.

2.4 Genotypic characterization, *agr* genotyping and PCR amplification of *S. aureus* toxin genes

S. aureus coa and species-specific *nuc* genes were amplified by uniplex PCR, while detection of *S. aureus agr* allele types (I – IV), enterotoxin genes (*sea-see*), ET genes (*eta* and *etb*) and TSST-1 gene (*tst*) were performed by

multiplex PCRs. The nucleotide sequences of all oligonucleotide primer sets used in this study and the predicted sizes of their respective PCR amplified products are listed in Table 1. PCR amplification was carried out on a PTC-100 ™ programmable thermal cycler (Peltier-Effect cycling, MJ, RESEARCH, INC, UK) in a total reaction volume of 25 µl consisting of 12.5 µl of Dream*Taq* ™ Green Master Mix (2X) (Fermentas, USA), 0.1 µl of 100 pmol of each primer (Sigma, USA), 2 µl of the DNA template and water nuclease-free up to 25 µl.

2.4.1 Detection of *S. aureus* genetic marker and specific virulence factor, *nuc* gene

A total of 37 PCR cycles were run under the following conditions: DNA denaturation at 94°C for 1 min, primer annealing at 55°C for 0.5 min and DNA extension at 72°C for 1.5 min. After the final cycle, the reaction was terminated by a final extension step at 72°C for 3.5 min (Brakstad et al., 1992).

2.4.2 Coagulase genotyping

The thermal profile involved an initial denaturation step at 94°C for 45 s, followed by 30 cycles of 94°C for 20 s, 57°C for 15 s and 70°C for 15 s with a final extension step at 72°C for 2 min (Hookey et al., 1998).

The numerical index of discrimination of *coa* gene typing was assessed according to the Hunter-Gaston formula (Hunter and Gaston, 1988) and calculated using the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1} n_j (n_j - 1)$$

D = Numerical index of discrimination

N = Total number of isolates in the sample population

s = Total number of types obtained

nj = Number of isolates belonging to the jth type

2.4.3 Identification of *agr* alleles

The amplification program was 1 cycle of 5 min at 94°C, 26 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C and finally 1 cycle of 72°C for 10 min (Gilot et al., 2002).

2.4.4 Detection of toxin genes (*sea-see, eta, etb* and *tst)*

The PCR program comprised an initial denaturation at 94°C for 5 min, followed by 35 cycles of amplification (denaturation at 94°C for 2 min, annealing at 57°C for 2 min and extension at 72°C for 1 min), ending with a final extension at 72°C for 7 min (Mehrotra et al., 2000).

All PCR products were stored in the cycler at 4°C until they were collected. Aliquots of all amplified PCR products (10 µL), along with a 100-bp molecular weight DNA ladder (Fermentas, USA) were subsequently separated by electrophoresis on 1.5% molecular biology grade agarose gel (Sigma, USA) stained with 0.5 µg/ml ethidium bromide (Sigma, USA) on a mini slab horizontal electrophoresis unit (Bio-Rad, USA) at 100 V for 30 min. DNA bands were visualized and photographed under an ultraviolet transilluminator (Spectroline, USA).

3. Results

3.1 Prevalence of *Staphylococcus* species in Sharkia province

Out of 141 samples collected from different areas in Sharkia province, 46 *Staphylococcus* species (32.62%) were recovered. On the basis of cultural and biochemical properties, 26 isolates (18.44%) were identified as *S. aureus* and 20 isolates (14.18%) from mastitic milk samples were presumptively classified as CoNS. Prevalence of *Staphylococcus* species in different samples collected from human and animal sources are presented in Table 2.

3.2 Phenotypic characterization of *Staphylococcus* species

Staphylococci were identified by conventional methods. All of the 46 strains grew on mannitol salt agar were Gram positive cocci, non-motile, non-spore forming, arranged in grape-like clusters, fermentative and catalase positive. They were identified as staphylococci. Among them, 26 strains were tube coagulase test positive, β-hemolytic and produced the characteristic golden yellow pigments, so they were considered as *S. aureus*, while the other 20 strains were identified as CoNS. Also, API 20 Staph was used for biotyping of 20 CoNS and 5 coagulase positive

staphylococci (CoPS). It revealed the following CNS biotypes: 6 (30%) of each *S. xylosus* and *S. sciuri*, 5 (25%) *S. lentus*, 2 (10%) *S. chromogens* and 1 (5%) *S. simulans*.
A total number of 15 *S. aureus* strains obtained from milk (5), sausage (1), burger (2), minced meat (1), urine (1), pus (3), sputum (1) and CSF (1) were randomly chosen from the available 26 isolates from various locations. They were tested using uniplex PCR for amplification of *S. aureus* species-specific *nuc* and *coa* genes and multiplex PCRs for detection of *S. aureus agr* allele types, SE, ET and *tst* genes to investigate a possible relationship between *agr* groups and the occurrence of toxin genes in *S. aureus* isolates.

3.3 Uniplex PCR for amplification of *nuc* gene
Amplification of the extracellular thermostable nuclease (*nuc*) gene as *S. aureus* species specific and virulence factor produced a PCR product of approximately 270 bp in all *S. aureus* isolates tested (100%), thus confirming their affiliation to the *S. aureus* species (Fig 1A). All the 15 *S. aureus* strains identified phenotypically were found to possess *nuc* gene indicating that PCR result for *nuc* detection was in agreement with those of conventional methods.

3.4 Coagulase genotyping
The identification of the 15 isolates as *S. aureus* could be confirmed also by PCR amplification of the *coa* gene and molecular typing of theses isolates was done on the basis of *coa* gene polymorphism. Results of phenotypic characterization of coagulase production using rabbit plasma were in complete accordance with the PCR findings of these isolates. Amplification of *coa* gene from the 15 *S. aureus* isolates produced 19 different PCR products. The sizes of these products ranged from 410 to 750 bp. Most of the isolates [11 out of 15 (73.33%)] produced a single band, whereas 3 isolates (20%) yielded 2 amplification products and only one isolate (6.67%) produced 3 amplicons. Majority of the isolates yielded PCR products ranged from 600-750 bp in size. Typical polymorphism of the *coa* gene are shown in Fig 1B.
According to *coa* gene polymorphism, the 15 *S. aureus* isolates were grouped into 15 different groups. The Numerical index of discrimination (D-value) based on PCR product sizes was 1, which is a very good index for discrimination. With a high discriminatory index value, this coagulase genotyping method proved to be useful, technically simple, reproducible, rapid and efficient molecular typing tool for better and reliable discrimination of *S. aureus* strains isolated from human and animal sources in Egypt.

3.5 *agr* genotyping
The *agr* specificity group was determined by a simple and rapid multiplex PCR method. The analysis of the PCR results showed that all analyzed strains possessed *agr* gene basing on the amplicon size difference (Fig 1C). Typing of the *agr* operon revealed the predominance of *agr* group I, being present in 14 out of 15 *S. aureus* isolated from all types of clinical samples (93.33%). On the other hand, only one strain from pus sample was found to harbor *agr* group III (6.67%) and none was positive for both *agr* types II and IV.

3.6 Detection of *S. aureus* exotoxin gene profiles
The occurrence of SE, ET and TSST genes was determined by multiplex PCRs. The sizes of the amplified PCR products from the representative toxigenic *S. aureus* strains corresponded to the predicted sizes as shown in Fig 1D and E. In the present study, all 15 *S. aureus* isolates tested were toxin producers. Of the five enterotoxin genes examined, only *sea* and *see* were identified. The most abundant toxin gene was *sea* as it presented in almost all isolates [13 out of 15 strains (86.67%)]. Consistently, *see* was reported to be the least frequently detected SE gene as it was amplified in only one *S. aureus* isolate from pus sample (6.67%). All the isolates were negative to the PCR assays targeting *seb*, *sec* and *sed* genes. With regard to the presence of ET genes in addition to the TSST-1 gene, both *eta* and *tst* genes were randomly distributed between the isolates, but none of the isolates harbored the gene encoding *etb*. The prevalence rates of *eta* and *tst* were 53.33% and 33.33%, respectively. It was found that all 5 strains possessed *tst* gene were simultaneously *sea* producers. With respect to different combinations of the recovered 4 toxin genes, 6 toxin gene patterns were found in 15 *S. aureus* isolates. The combination of *sea* and *eta* showed the highest prevalence (33.33%).
A summary of data on the distribution of the virulence determinant genes and *agr* allotypes among 15 clinical *S. aureus* isolates collected from various clinical samples by PCR are shown in Table 3 and the characteristics of the these 15 *S. aureus* isolates are presented in Table 4.

3.7 Association between *agr* groups and toxin gene profiles of *S. aureus* isolates
An important phenotypic feature of the *agr* system is the exoprotein profile; all *agr* positive strains in the present study produce exotoxins. The 6 toxin gene patterns found in 15 *S. aureus* isolates were classified into *agr* groups I

and III. The *agr* I genotype strains were found to possess higher capabilities of producing exotoxins and more likely to harbor *sea* gene as almost all *agr* I isolates (86.67%) were *sea* producers, while the only *agr* type III strain isolated from pus sample harbored *see* gene. Moreover, our *tst* positive strains were associated with *agr* type I. Since diverse toxin gene patterns were found in the strains belonging to the same *agr* group, toxin gene typing could be more discriminatory than *agr* typing.

Table 1 Nucleotide sequences and predicted sizes of PCR products for *S. aureus* oligonucleotide primers used in this study.

Gene	Primer	Oligonucleotide sequence (5′-3′)	Size of amplified product (bp)	References
nuc	nuc F nuc R	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	270	Brakstad et al., 1992
coa	Coa F Coa R	ATA GAG ATG CTG GTA CAG G GCT TCC GAT TGT TCG ATG C	Variable	Hookey et al., 1998
agrI	Pan agr1	ATG CAC ATG GTG CAC ATG C GTC ACA AGT ACT ATA AGC TGC GAT	441	Gilot et al., 2002
agrII	Pan agr2	ATG CAC ATG GTG CAC ATG C TAT TAC TAA TTG AAA AGT GGC CATAGC	575	Gilot et al., 2002
agrIII	Pan agr3	ATG CAC ATG GTG CAC ATG C GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G	323	Gilot et al., 2002
agrIV	Pan agr4	ATG CAC ATG GTG CAC ATG C CGA TAA TGC CGT AAT ACC CG	659	Gilot et al., 2002
sea	GSEAR-1 GSEAR-2	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	102	Mehrotra et al., 2000
seb	GSEBR-1 GSEBR-2	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	164	Mehrotra et al., 2000
sec	GSECR-1 GSECR-2	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG	451	Mehrotra et al., 2000
sed	GSEDR-1 GSEDR-2	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC	278	Mehrotra et al., 2000
see	GSEER-1 GSEER-2	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	209	Mehrotra et al., 2000
eta	GETAR-1 GETAR-2	GCAGGTGTTGATTTAGCATT AGATGTCCCTATTTTTGCTG	93	Mehrotra et al., 2000
etb	GETBR-1 GETBR-2	ACAAGCAAAAGAATACAGCG GTTTTTGGCTGCTTCTCTTG	226	Mehrotra et al., 2000
tst	GTSSTR-1 GTSSTR-2	ACCCCTGTTCCCTTATCATC TTTTCAGTATTTGTAACGCC	326	Mehrotra et al., 2000

nuc, Nuclease; *coa*, Coagulase; *agr*, Accessory gene regulator; *eta* and *b*, exfoliative toxin a and b; *sea-e*, staphylococcal enterotoxin a-e; TSST, toxic shock syndrome toxin.

Table 2 Prevalence of *Staphylococcus* species in different samples collected from animal and human sources in Sharkia province.

Sample type	Number of samples analyzed	Number of samples (%) showing *S. aureus*	Number of samples (%) showing CoNS
Mastitic milk	**64**	**7 (10.94)**	20 (31.25)
Meat products	**32**	**7 (21.88)**	0 (0)
Sausage	7	3 (42.86)	0 (0)
Burger	11	3 (27.27)	0 (0)
Minced meat	14	1 (7.14)	0 (0)
Human subjects	**45**	**12 (26.67)**	0 (0)
Urine	8	2 (25)	0 (0)
Pus	7	4 (57.14)	0 (0)
Sputum	6	2 (33.33)	0 (0)
CSF	6	1 (16.67)	0 (0)
Blood	7	1 (14.29)	0 (0)
Pericardial fluid	5	1 (20)	0 (0)
Peritoneal fluid	6	1 (16.67)	0 (0)
Total	**141**	**26 (18.44)**	**20 (14.18%)**

CSF: Cerebrospinal fluid

Table 3 Distribution of the virulence determinant genes and *agr* allotypes among 15 *S. aureus* isolates collected from various clinical samples.

Source of *S. aureus* isolates (*)	Number (%) of isolates positive for virulence determinant genes						Number (%) of isolates harboring a particular *agr* allotype	
	sea	***see***	***eta***	***tst***	***coa***	***nuc***	***agr* I**	***agr* III**
Mastitic milk (5)	4	0	3	1	5	5	5	0
Sausage (1)	1	0	0	1	1	1	1	0
Burger (2)	2	0	2	1	2	2	2	0
Minced meat (1)	1	0	1	0	1	1	1	0
Urine (1)	1	0	0	0	1	1	1	0
Pus (3)	2	1	1	2	3	3	2	1
Sputum (1)	1	0	1	0	1	1	1	0
CSF (1)	1	0	0	0	1	1	1	0
Total (15)	**13 (86.67%)**	**1 (6.67%)**	**8 (53.33%)**	**5 (33.33%)**	**15 (100%)**	**15 (100%)**	**14 (93.33%)**	**1 (6.67%)**

* Number of isolates
CSF: Cerebrospinal fluid

Table 4. Characteristics of 15 *S. aureus* isolates collected from different samples.

Isolates code number	Source	*coa* (*)	*nuc*	*agr* class	SE genes	ET genes	*tst*
1 M	Milk	2- 506, 581	+	I	*sea*	*eta*	-
2 M	Milk	1- 636	+	I	*sea*	-	+
3 M	Milk	1- 588	+	I	*sea*	-	-
4 M	Milk	3- 477, 627,693	+	I	-	*eta*	-
5 M	Milk	1- 578	+	I	*sea*	*eta*	-
1 Sa	Sausage	1- 643	+	I	*sea*	-	+
1 B	Burger	1- 750	+	I	*sea*	*eta*	+
2 B	Burger	1- 742	+	I	*sea*	*eta*	-
1 Mm	Minced meat	1- 725	+	I	*sea*	*eta*	-
1 U	Urine	2- 470, 621	+	I	*sea*	-	-
1 P	Pus	1-650	+	I	*sea*	*eta*	+
2 P	Pus	1- 621	+	I	*sea*	-	+
3 P	Pus	1- 441	+	III	*see*	-	-
1 Sp	Sputum	1- 556	+	I	*sea*	*eta*	-
1 CSF	CSF	2- 410, 600	+	I	*sea*	-	-

M, Milk; B, Burger; Sa, Sausage; Mm, Minced meat; P, Pus; U, Urine; Sp, Sputum; CSF, Cerebrospinal fluid**.**
* First digit indicates number of bands; subsequent values indicate the molecular weight of each band.

4. Discussion

S. aureus has been recognized as a major pathogen in human and animal infections. An accessory gene regulator (*agr*) locus is known to be a global regulatory system that controls the coordinated expression of a spectrum of extracellular toxins and virulence factors produced by this organism (Novick, 2003).

In the present study, 27 staphylococcal isolates were isolated from 64 mastitic milk samples (42.19%). In mastitc animals, the prevalence of staphylococci differed among countries such as 93.16% in India (Sentitula et al., 2012), 40% in Zimbabwe (Kudinha and Simango, 2002) and 33% in Pakistan (Arshad et al., 2006). Phenotypic identification including coagulase production, pigmentation on milk agar and hemolytic activity onto blood agar classified the staphylococcal isolates into *S. aureus* and CoNS. In this work, *S. aureus* was found in 7 mastitic milk samples (10.94%). Similar results were observed in an earlier study carried out in Germany (Tenhagen et al., 2009). In Ireland and Bangalore, the prevalence rates of *S. aureus* in milk samples from dairy cattle with mastitis were 23% and 24%, respectively (Keane et al., 2013; Sumathi et al., 2008) which are much higher than the frequency observed in this study. Therefore, a comparison of the results of the present study and those reported by other authors is difficult because the occurrence of *S. aureus* as a causative agent of mastitis varies according to the area, handling practices of the animals and hygienic conditions during milking (Jayarao et al., 2004). CoNS play a major role in causing bovine mastitis, since they were isolated in a high percentage (31.25%). This observation is in harmony with that in a study carried out in Netherlands (Sampimon et al., 2009). However, lower isolation rates of CoNS were observed in milk samples from clinical mastitis cases in Ireland (4%), Zimbabwe (22.9%) and Germany (16.4%) (Keane et al., 2013; Kudinha and Simango, 2002; Tenhagen et al., 2009). The API 20 Staph in the current study revealed the following biotypes: 6 (30%) of each *S. xylosus* and *S. sciuri*, 5 (25%) *S. lentus*, 2 (10%) *S. chromogens* and 1 (5%) *S. simulans*. The noticed results are partially similar with data from scientific literatures which isolated *S. xylosus* and *S. chromogens* from clinical mastitis cases in Zimbabwe with percentages of 40% and 13.3%, respectively (Kudinha and Simango, 2002) and recovered *S. simulans* from mastitic cow milk in Romania with a percentage of 4.5% (BrÎnda et al., 2010). However, a lower isolation rate (13.7%) of both *S. sciuri* and *S. lentus* associated with mastitis cases in Romania was recovered (BrÎnda et al., 2010).
In the present study, *S. aureus* was isolated from meat products with an incidence rate of 21.88% which is lower than that in Netherlands (45.57%) (van Loo et al., 2007).

In addition, Incidence of *S. aureus* from samples from human sources is agreeable with the earlier research report in India (Nadig et al., 2006). The highest numbers of *S. aureus* were obtained from pus, followed by sputum and urine. This finding is exactly in conformity with those reported in Nigeria (Fayomi et al., 2011). Also, majority of *S. aureus* isolates in India were recovered from pus samples (Dar et al., 2006; Nadig et al., 2006). Meanwhile, lowest

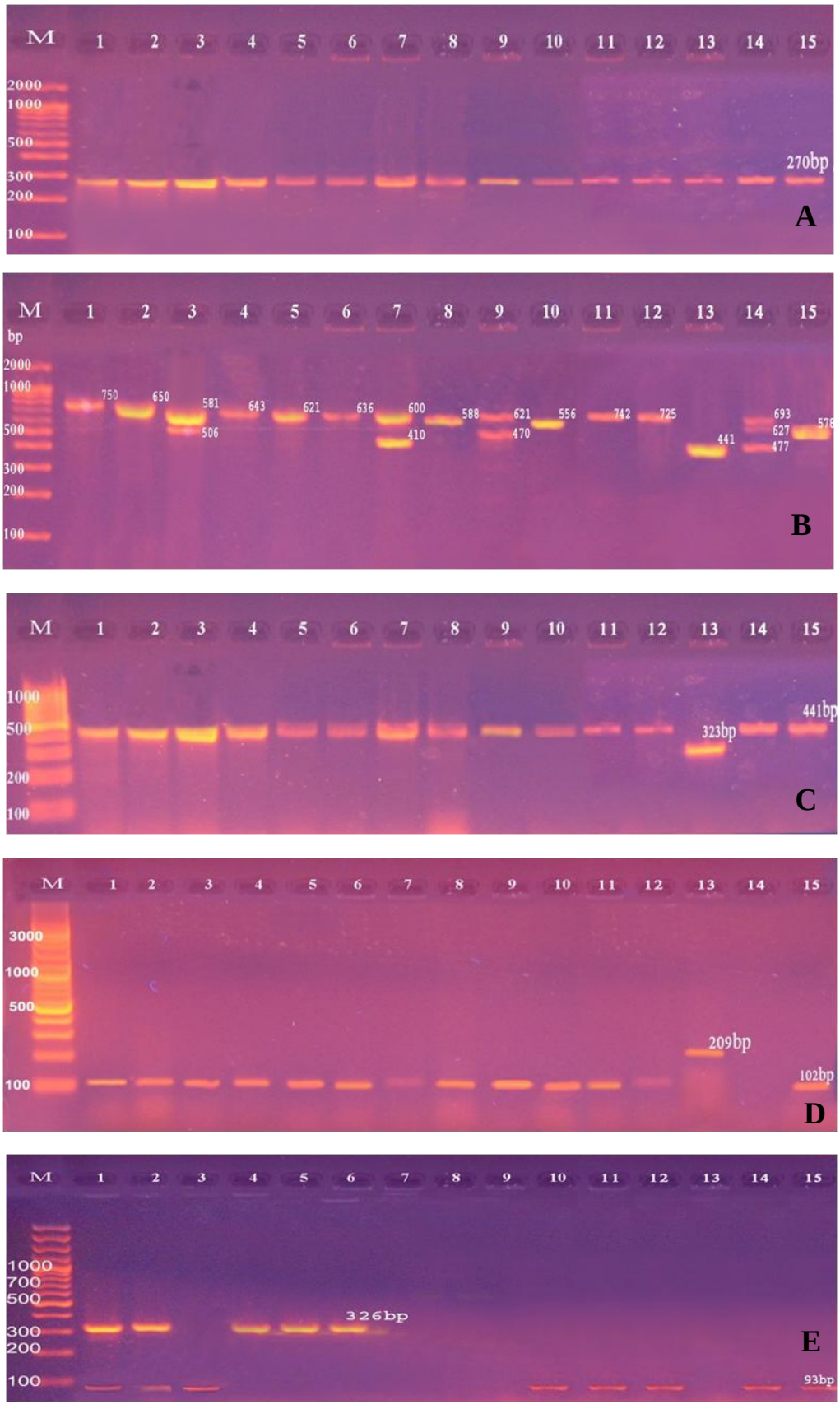
incidence rates of *S. aureus* were observed in blood, CSF, peritoneal and pericardial fluid samples compared to earlier reports in North India, Nigeria and Lahore (Dar et al., 2006; Fayomi et al., 2011; Hafeez et al., 2004).
In the current study, all 15 *S. aureus* isolates identified phenotypically were confirmed genotypically by uniplex PCR targeting *nuc* and *coa* genes. Comparable PCR-based systems for identification of *S. aureus* isolates have been used (Brakstad et al., 1992; Hookey et al., 1998). The *nuc* primer set recognized all *S. aureus* identified by conventional methods. These results substantiate those obtained by other methodological approaches (Brakstad et al., 1992; Costa et al., 2005). It determined the generation of a PCR product of approximately 270 bp for all *S. aureus* isolates as was previously reported (Brakstad et al., 1992). The PCR products of the genes encoding coagulase investigated in the present work displayed typical gene polymorphism. This attribute allowed a genotypic characterization of the isolates (Hookey et al., 1998). In the current study, most of the isolates (11) produced a single band, whereas 3 isolates yielded double-banded amplification product and only 1 isolate produced 3 amplicons. These results are consistent with previous reports explaining the presence of double-banded amplification products with different allelic forms of the *coa* gene (da Silva and da Silva, 2005; Goh et al., 1992). Calculation of the discriminatory power of *coa* gene typing yielded a high D index value (1) which is much greater than that in a previous report in South India (0.55) (Himabindu et al., 2009).

During this study, we have investigated the prevalence of *agr* specificity groups among 15 *S. aureus* strains by multiplex PCR which revealed that all analyzed strains harbored *agr* gene. Typing of the *agr* operon revealed that all *S. aureus* genotypes could be assigned to two major *agr* allelic groups. Most of the isolates were belonged to *agr* group I (93.33%). These findings were commonly reported by other investigators in China and Tehran (He et al., 2013; Peerayeh et al., 2009). On the other hand, the absence of *agr* types II and IV in our isolates differs from data described in a previous published paper in Tehran (Peerayeh et al., 2009). This could reflect ecological and geographical differences. With regards to detection of genes encoding SEs, ETs and TSST-1 by multiplex PCR technology, 14 out of 15 *S. aureus* isolates (93.33 %) were enterotoxigenic. This prevalence rate was noticeably higher than that detected previously in *S. aureus* from human, food and animal sources in Malaysia (20.8%) (Lim et al., 1982). The predominant classical SE gene varied from country to country: Germany, *sea*, *sec* (Becker et al., 2003); New Zealand, *seb* (Boerema et al., 2006); Poland, *sec* (Bania et al., 2006) and here in Egypt, *sea*. Differences in the nature of the study population and in PCR screening methodologies (primers, amplification cycles and single versus multiplex PCR) may contribute to such variations. Interestingly, our detection rate for *eta* gene (53.33%) was noticeably higher than that of other investigators in Germany and Turkey (Becker et al., 2003; Demir et al., 2011). Moreover, all 5 strains possessed *tst* gene in the present study were simultaneously *sea* producers. Similar coexisting gene combination was also reported in the Czech Republic (Hrstka et al., 2006). Ironically, none of the isolates undertaken in this study possessed *seb*, *sec*, *sed* and *etb* genes. Similarly, Malaysian *S. aureus* strains did not harbor these genes (Lim et al., 2012). Considering the association between *agr* groups and toxin production, all *sea* and *tst* positive strains were of *agr* type I. This finding is in agreement with another observation reported in Malaysia (Lim et al., 2012). Finally, in agreement with another study in China (Xie et al., 2011), our results showed that the high diversity of toxin gene patterns of *S. aureus* isolates could be used as a discriminatory method for genotyping *S. aureus* strains.

Figure 1: Agarose gel electrophoresis patterns showing typical amplification products in uniplex PCR for *nuc* (A) and *coa* (B) genes and in multiplex PCRs for *agr* specific groups (C), SE genes (D) and ET and TSST-1 genes (E) of DNA from 15 *S. aureus* isolates from various samples. Lane M, DNA molecular size marker (100-bp); lanes 1, 11, burger samples; lanes 2, 5, 13, pus samples; lanes 3, 6, 8, 14, 15, milk samples; lane 4, sausage sample; lane 7, cerebrospinal fluid sample; lane 9, urine sample; lane 10, sputum sample and lane 12, minced meat sample.

Fig 1A lanes 1-15, *S. aureus* isolates showing *nuc* positive PCR band.
Fig 1B lanes 1-15, different sizes of *S. aureus* coagulase PCR products.
Fig 1C lanes 1-12, 14, 15, *agr* I positive isolates; lane 13, *agr* III positive isolate.
Fig 1D lanes 1-12, 15, *sea* positive isolate; lane 13, *see* positive isolate.
Fig 1E lanes 1, 2, *eta* and *tst* positive isolates; lanes 3, 10, 11, 12, 14, 15, *eta* positive isolates; lanes 4-6, *tst* positive isolates.

Amplification products and DNA fragment sizes are marked in base pairs (bp) on the right of the bands.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
2000
1000
500
300
200
100
270bp
A
bp
750 650 581 643 621 636 600 588 621 556 742 725 693 627 578
506 410 470 441 477
B
1000
500
200
100
323bp
441bp
C
3000
1000
500
100
209bp
102bp
D
1000
700
500
300
200
100
326bp
93bp
E

5. Conclusion

In summary, data presented in this molecular epidemiological study showed a broad distribution of different *S. aureus* and CoNS strains from human and animal sources in Egypt and provided updated data on the correlation between *agr* genotypes and the toxin gene content of *S. aureus*. Our PCR results showed that all analyzed strains, regardless of the source of isolation harbor the *agr* gene, showing that they have a functional QS system and it is noteworthy that all *agr* positive strains produce exotoxins. Moreover, all *sea* and *tst* positive strains were of *agr* type I. Another point is existence of diverse toxin gene patterns and *coa* gene polymorphism in the strains belonging to the same *agr* group indicates that toxin and coagulase genotyping could be more discriminatory than *agr* typing. This can allow the deciphering of important epidemiological information about *S. aureus* clinical isolates in Egypt which is a prerequisite for monitoring and limiting the occurrence and spread of *S. aureus* infections.

6. References

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