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Genotypic Molecular Detection of Certain Genes Encoding Virulence Determinates and Antibiotic Resistance in *Staphylococcus Aureus* Isolates from Mastitis Cows

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ABSTRACT

A total of 10Staphylococcusaureusisolates were recovered from 117 milk samples which were obtained from 93 dairy cows indifferent farms suffered from clinical mastitis, these isolates were phenotypically characterized and identified based on colony morphology andbiochemical tests. In addition, antibiotic susceptibility pattern of these strains was investigated and a trial to investigate the mechanism of the isolated bacterium to antibiotic resistance by PCR detection of class 1 integron. The milk samples were examined bacteriologically: Thirty-three out of 117 samples (28.2%) were positive for staphylococcus. Ten out of 33 samples (30.3%) were coagulase positive staphylococcus. The S. aureus isolates were highly sensitive to Cephalothin (DF30), Ampicillin/sulbactam (SAM), Methicillin(DP5µg), Amoxicillin-Clavulanic acid (AMC30) and Trimethoprime-Sulphamethaxole (SXT25µg), Susceptibility rates of S. aureus to these antibiotics were: 90, 80, 80, 70 and70%, respectively. Higher resistance was observed to Streptomycin (S10µg), Kanamycine (K30µg), Tetracycline (Te30µg), Erythromycin (E15µg), Cefotasime (CTX30µg), Cefoperazone (CFP75µg), Ampicillin (AMP10µg), Gentamycine (GN10µg), Amoxicillin (AX25µg) and Ciprofloxacin(CIP5µg), Susceptibility rates of S. aureus to these antibiotics were: 30, 30, 30, 30, 40, 40, 40, 40,40 and 50%, respectively. The molecular detection of S. aureus specific sequence gene*(Sa)*, genes encoding clumping factor A *(clfA)*, gene encoding S. aureus protein A *(spa)*and the class 1 integron using Polymerase Chain Reaction were carried out. A total of 10 Staphylococcus aureus isolates were negative for the presence of class 1 integron but yielded an amplicon size of about107 bp in case of S. aureus specific sequence gene *(Sa)*, an amplicon size of about 638bp in case of*clfA* gene. Amplification of the*(spa)* gene encoding S. aureus protein A was detected in 9 strains out of 10 (90%)and yielded an amplicon size of about 226bp. The data in this study provided an overview on the incidence of staphylococcus aureus in clinical mastitis and the distribution of virulence determinants and antibiotic resistance of these S. aureus strains.

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1. INTRODUCTION

S. aureus is a 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 (Delbes et al., 2006). It may cause a spectrum of pathologies in humans and animals (Viana et al., 2007). Mastitis is one of the major causes of economic losses in dairy industry worldwide, with a wide variety of microorganisms involved. The major microorganism is Staphylococcus aureus. *S. aureus* is often responsible for intramammary infections in bovines, and is the main etiological agent of contagious mastitis in dairy herds (Gilbert et al., 2006andTaverna et al., 2007). *S.aureus* has a capacity to produce a large number of putative virulence factors (Kalorey et al., 2007). Some of these factors may be of more importance than others indifferent diseases or at different stages of the pathogenesis of particular infections, as not all factors are produced by each strain. Indiscriminate use of existing antibiotics leads to proliferation of antibiotic resistance and poses a dilemma for the future treatment of bacterial infection. Antibiotic resistance in microbes still remains one of the leading concerns in global public health, and several mechanisms involving mobile genetic elements have been shown to contribute to the wide spread and distribution of antibiotic resistant genes among bacteria. In recent years, the role of integrons as a mobile genetic mechanism in horizontal transfer of antibiotic resistance has been well established. As a novel antibiotic resistance mechanism, class 1 integron has been identified as a primary source of antimicrobial resistance genes in Gram-negative

organisms. However, most available occurrence of integrons had been limited within Gram-negative microbes, very little is known for clinical Gram-positive bacteria (Xuet al., 2011). This study aimed to throw the light on the incidence of staphylococcus aureus in clinical mastitis, the genotypic characterization of S. aureus strains isolated from dairy cows suffering from mastitis and provided an overview on the distribution of virulence determinants of these S. aureus strains which contribute in bovine mastitis problem in the Egyptian farms. This study also aims to concentrate on the latest development of class 1 integron in S. aureus isolates, including summary of prevalence and occurrence of class 1 integron, analysis of correlation between integron and antibiotic resistance, further demonstration of the role integrons play as antibiotic determinants, as well as origin and evolution of integron-associated gene cassettes.

2. MATERIAL AND METHODS

2.1. Animal

A total number of (93) lactating cows showing signs of clinical mastitis were included in this study from different private and governmental farms in El Bohera Governorate, Egypt.

2.2. Collection of clinical mastitis milk samples

A total number of 117 milk samples were collected from lactating cows before the animal have been treated with either intramammary or systemic antimicrobial agents. All milk samples were collected according to *(Quinn et al., 2002).* The udder of each animal was palpated before sampling for presence of clinical signs of mastitis, the examined udders were thoroughly washed and carefully dried with clean dry towel. Then the teat was swabbed with 70% ethyl alcohol. After that the first few jets of milk were discarded and milk samples were collected in 50ml sterile falcon tube from clinically affected quarter. All samples were taken during the period of spring (33samples) and summer (84samples) of 2014.

2.3. Bacteriological examination of the clinical mastitis milk samples

Isolation of S. aureus from raw milk (Quninn et al., 2002):

The collected milk samples on the brain heart infusion broth were incubated at 37 ^{o}C for 18–24 hours then with centrifuged at 3000 rpm for 20 minutes. (The cream and supernatant fluid were discarded). Dry heat fixed films were done from the sediment. The smears were stained with Gram's stain used and examined under oil immersion lens for the identification of the microorganisms to detect the suggestive bacterial growth. The presence of non-capsulated Gram-positive cocci which tend to be arranged in irregular cluster, considering this sample suspected to be a staphylococcal infection and then subjected to the second step of examination.

A loopful from the sediment of each sample was plated onto nutrient agar, mannitol salt agar (selective media for Staphylococcus Sp.) and blood agar media *(Quinn et al., 1994)* according to *(Cruickshank et al., 1975).* As well as onto the Baired Parker's agar medium *(Baired-Parker, 1962).* The inoculated plates were incubated at 37 ^{o}C for 24–48 hours under aerobic conditions. Suspected colonies were picked up for purification and subjected for identification microscopically and biochemically according to *(Collee et al., 1996, Quinn et al., 2002, Boerlin et al., 2003*and*de Freitas et al 2013).*

2.4. Antibacterial susceptibility test

Antimicrobial susceptibility is according to *Jan Hudzicki, (2013)* and was performed by disk diffusion agar method *(C.L.S.I, 2012).*

2.5. Extraction of DNA (QIAamp® DNA Mini and Blood Mini Handbook)

According to QIAamp DNA mini kit instructions, 20 µl QIAGEN protease were pipetted into the bottom of a 1.5 ml microcentrifuge tube, 200µl of the sample was added, 200µl buffer AL were added to the sample, mixed by pulse vortexing for 15 seconds. The mixture was incubated at 56˚C for 10 min, the 1.5 ml microcentrifuge tube werecentrifugated to remove drops from the inside of the lid, 200 µl ethanol (96%) were added to the sample, and mixed again by pulse vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifugated to remove drops from the inside of the lid. The mixture from step 6 was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim,the cap was closed, and centrifugated at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded, the QIAamp mini spin column was carefully opened and 500 ml buffer AW1 were added without wetting the rim, the cap was closed, and centrifugated at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded,the QIAamp mini spin column was carefully opened and 500 ml buffer AW2 were

added without wetting the rim, the cap was closed, and centrifugated at full speed for 3 min. The QIAamp mini spin column was placed in a new 2 ml collection tube and the old collection tube was discarded with the filtrate. Centrifugation at full speed for 1 min was done. The QIAamp mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 100 µl buffer AE were added. The QIAamp mini spin column was incubated at room temperature (15-25˚C) for 1 min, and then centrifugated at 8000 rpm for 1 min.

2.6. PCR Amplification

For PCR amplification, a reaction mixture of (25µl) contained12.5µlEmeraldAmpGTPCR mastermix(2x premix),4.5µl of PCR grade water,1µl offorward primer (20 pmol), 1µl of reverse primer (20 pmol) and 6µl of template DNA(Lee et al., 2012).

Table 1: Interpretation of the results of the antimicrobial susceptibility assay:

		Inhibitory Zone Diameter		
Antimicrobial Agent	Code disc	resistant	intermediate	sensitive
Ampicillin	AMP10	⩽13	14 - 16	⩾17
Amoxicillin	AX25	⩽22	23 - 30	⩾31
Ampicillin/sulbactam	SAM	⩽11	12 - 14	⩾15
Cefoperazone	CFP75	⩽15	16 - 18	⩾19
Ciprofloxacin	CIP5	⩽15	16 - 20	⩾21
Cefotasime	CTX30	⩽14	15 - 22	⩾23
Trimethoprime-Sulphamethaxole	SXT25	⩽10	11 - 15	⩾16
Erythromycin	E15	⩽13	14 - 22	⩾23
Gentamycine	GN10	⩽12	13 - 14	⩾15
Streptomycin	S10	⩽11	12 - 14	⩾15
Tetracycline	Te30	⩽14	15 - 18	⩾19
Methicillin	DP5	⩽9	10 - 13	⩾14
Kanamycine	K30	⩽13	14 - 17	⩾18
Amoxicillin-Clavulanic acid	AMC30	⩽19	20 - 24	⩾25
Cephalothin	DF30	⩽14	15 - 17	⩾18

Table 2: Primers for amplification of the Staphylococcal genes:

Target gene	Primer sequence (5'-3')	Length of amplified product (bp)	Reference
S. aureus specific sequence gene *(Sa)*	F 5′- AAT CTT TGT CGG TAG ATA TTC TTC ACG-3′	107bp	*Martineau et al., 1998*
	R 5′- CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA-3′		
clfA	F5′-GCAAAATCCAGCACAACAGGAAACGA-3′	638bp	*Mason et al., 2001*
	R 5 ′-CTTGATCTCCAGCCATAATTGGTGG-3′		
spa	F5′-TCA ACA AAG AAC AAC AAA ATG C-3′	226bp	*Wada et al., 2010*
	R 5 ′-GCT TTC GGT GCT TGA GAT TC-3′		
Integron class - 1	F5′-GCATCCAAGCAGCAAG-3′	Variable	*Ahmed et al., 2007*
	R 5 ′-AAGCAGACTTGACCTGA-3′		

The reaction tubes were subjected to thermal cycling with the same programme described previously by *(Martineau et al., 1998)* for *(Sa)*, *(Mason et al., 2001)* for *clfA*, *(Wada et al., 2010)* for spa and *(Ahmed et al., 2007)* for Integron class – 1.

DNA Molecular weight marker used areGel Pilot 100 bp ladder (cat. no. 239035) supplied from QIAGEN, number of bands: 6, size range: 100-600 bp. And Gel Pilot 100 bp plus ladder (cat. no. 239045) supplied from QIAGEN (USA), number of bands: 11, size range: 100-1500 bp. The ladder was mixed gently by pipetting up and down. 6 µl of the required ladder were directly loaded.

Electrophoresis grade agarose (1.5 g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70˚C, then 0.5µg/ml ethedium bromide was added and mixed thoroughly. The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization. The comb was then removed, and the electrophoresis tank was filled with TBE buffer. Twenty µl of each PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software (Sambrook et al., 1989).

3. RESULTS

Isolation and identification of microbe:

Morphological identification:

Out of 117 milk samples of clinically bovine mastitis 35isolates (30%)tested positive for Staphylococcus on the basis of morphology through Gram's stained films, they revealed spherical cocci 0.8 – 1 µm in diameter. Films prepared from solid media showed grape-like clusters vary in size with some single or paired cocci. From broth cultures, small groups, pairs and short chains of less than five cocci in a line, could be observed. The organism was non-capsulated Gram-positive, non-sporing and non-motile.

Cultural characteristics:

After aerobic incubation on nutrient agar, mannitol salt agar for 24-48 hours at 37 ^{o}C, colonies suspected as Staphylococcus were large, 1– 3 mm in diameter, and well isolated colonies reached 4 mm in diameter. The suspected colonies were round, convex, smooth with glistening surface. Pigmentation of the colonies varied from cream-buff to golden yellow or even orange coloration. The observation of pigmentation made it possible provisional identification of colonies suspected as Staphylococcus colonies in mixed cultures. Out of the cultivated 117 samples, 33 showed pigmentation with an incidence of (28.2%). After aerobic incubation out of these 33 isolates on Baired-Parker's agar media for 24-48 hours at 37 ^{o}C,10 isolates(30.3%) produced black, shiny, convex colonies with entire margins and clear zone surrounding the colonies with or without an opaque zone. The observation of colonies made it possible provisional identification of S. aureus. This result confirmed by biochemical identification of 33 suspected Staphylococcus isolates that they were 10 isolates (30.3%)were positive for Catalase test (slide technique), Oxidase test, Oxidation - fermentation of glucose (o-f test), Urease test, Gelatin liquefaction test, Mannitol fermentation test, Coagulase test and showed β-hemolysis on Nutrient agar containing 7.5% NaCl and 5% (V/V) defibrinated sheep blood was used. Plates were inoculated on the surface, incubated for 24 hours.

Results of Antibiotic susceptibility test:

Antibiotic resistance testing revealed that among the 10isolates, one was resistance to all the antibiotics used in this study. Cephalothin (DF30), Ampicillin/sulbactam(SAM), Amoxicillin-Clavulanic acid(AMC30), Trimethoprime-Sulphamethaxole(SXT25) and Methicillin(DP5) were the most effective antibiotics for S. aureus strains. Susceptibility rates of S. aureus to these antibiotics were: 90% for Cephalothin (DF30),80% for Ampicillin/sulbactam (SAM) and for Methicillin (DP5) and 70% for trimethoprim-sulphamethoxazole(SXT25) and for Amoxicillin-Clavulanic acid (AMC30). Higher resistance was observed to Streptomycin (S10), Kanamycine (K30),Tetracycline (Te30), Erythromycin(E15), Cefotasime(CTX30), Cefoperazone(CFP75), Ampicillin (AMP10), Gentamycine(GN10), Amoxicillin (AX25) and Ciprofloxacin(CIP5), Susceptibility rates of S. aureus to these antibiotics were: 30, 30, 30, 30, 40, 40, 40, 40,40 and 50%, respectively.

Results of amplification of target genes:

Amplification of S. aureus specific sequence gene *(Sa)*resulted in a single amplicon with size of approximately 107 bp for all isolated strains, which confirmed the assumption that all the strains were Staphylococcus aureus, indicating no size polymorphisms of this gene (Figure 1). Amplification of the clumping factor A*(clfA)* gene

resulted in a single amplicon with a size of approximately 638 bp for all 10 S. aureus strains indicating no size polymorphisms of this gene (Figure 2). Amplification of S. aureus protein A gene *spa* resulted in a single amplicon with a size of approximately 226bp for 9 S. aureus strains out of 10 (90 %) (Figure3). All samples exhibited a negative PCR reaction with the Integron class -1gene (Figure 4).

Table 3: Antibiotic sensitivity test for the 10 S. aureus isolates:

		Sensitive		Intermediate		resistant	
Antimicrobial Agent	**Code disc**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Cephalothin	DF30	9	90%	1	10%	0	0
Ampicillin/sulbactam	SAM	8	80%	0	0	2	20%
Methicillin	DP5	8	80%	0	0	2	20%
Trimethoprime-Sulphamethaxole	SXT25	7	70%	1	10%	2	20%
Amoxicillin-Clavulanic acid	AMC30	7	70%	1	10%	2	20%
Ciprofloxacin	CIP5	5	50%	0	0	5	50%
Amoxicillin	AX25	4	40%	0	0	6	60%
Gentamycine	GN10	4	40%	0	0	6	60%
Ampicillin	AMP10	4	40%	0	0	6	60%
Cefoperazone	CFP75	4	40%	0	0	6	60%
Cefotasime	CTX30	4	40%	1	10%	5	50%
Erythromycin	E15	3	30%	0	0	7	70%
Tetracycline	Te30	3	30%	0	0	7	70%
Kanamycine	K30	3	30%	1	10%	6	60%
Streptomycin	S10	3	30%	1	10%	6	60%

10	9	8	7	6	Pos	L	5	4	3	2	1	Neg

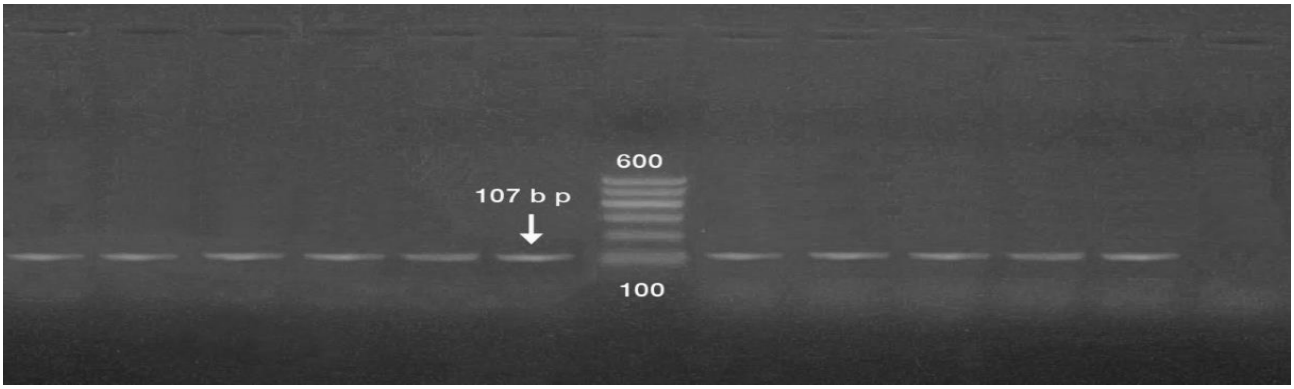

Figure (1): Typical amplicons of theS. aureus specific sequence gene *(Sa)*.
L: is 100 bp plus ladder (QIAGEN USA) used as size marker.
Pos: is control positive.Neg: is control negative.

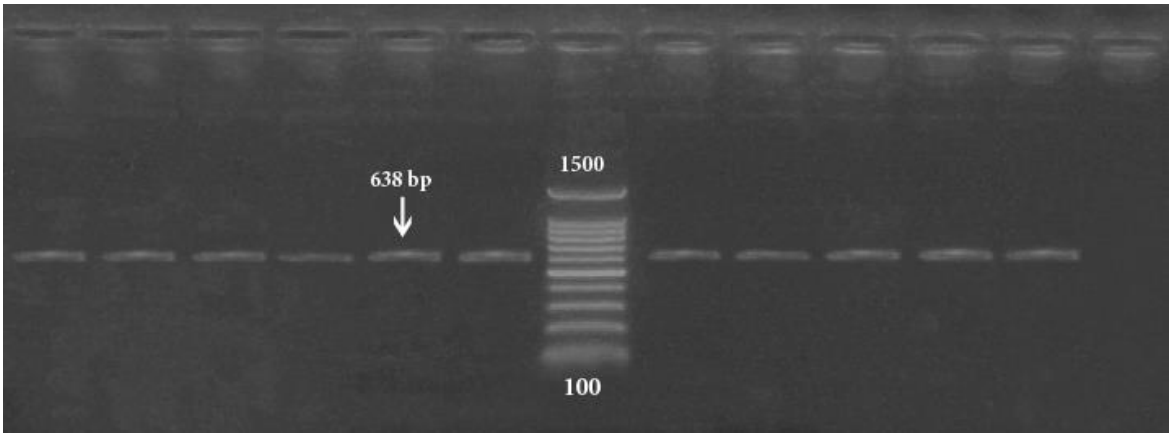

Figure (2): Typical amplicons of the clumping factor *(clfA)* gene in S. aureus. Isolate.
L is 100 bp plus ladder (QIAGEN USA) used as size marker.
Pos: is control positive. Neg: is control negative.

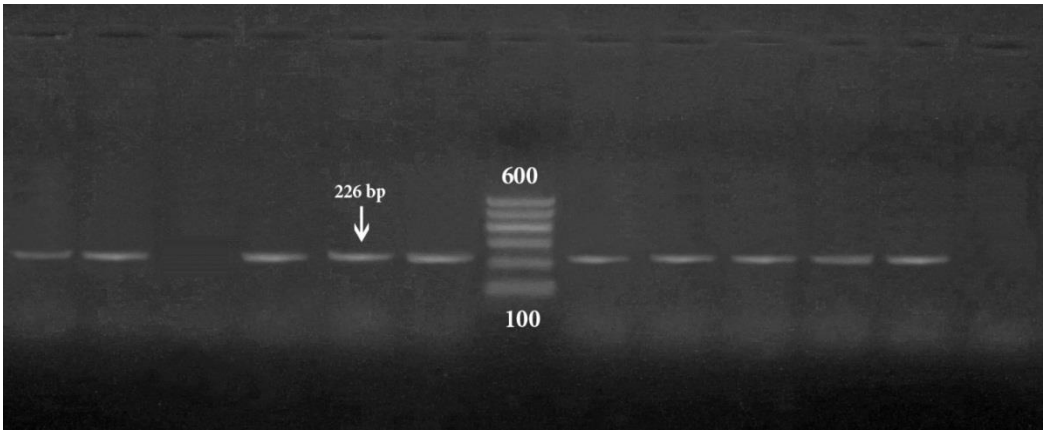

Figure (3): Typical amplicons of theS. aureus protein A gene *spa* gene in *S. aureus*. Isolate.
L is 100 bp plus ladder (QIAGEN USA) used as size marker.
Pos: is control positive. Neg: is controlnegative.

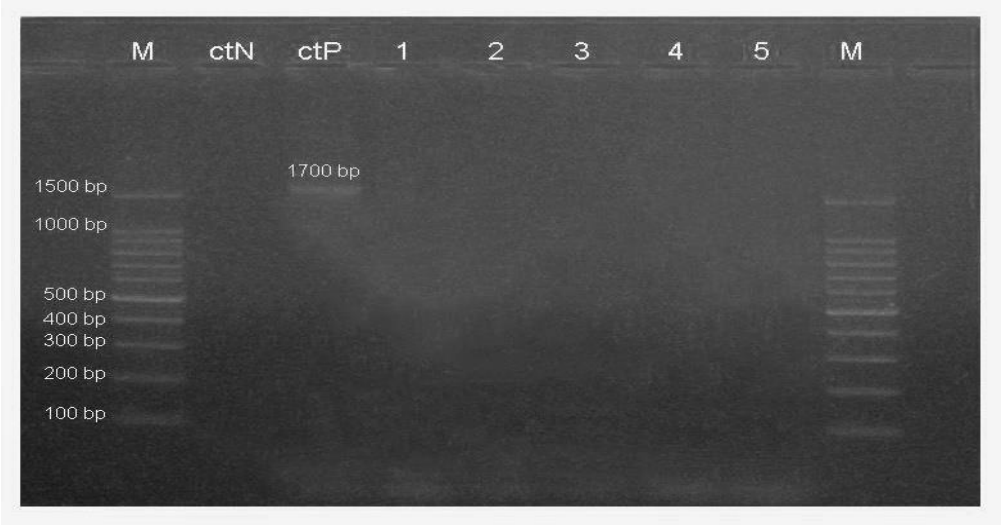

Figure (4)**:** All samples exhibited a negative PCR reaction with the Integron class -1 gene.
M: is a 100 bp ladder used as size marker.
CtP: is control positive. CtN: is control negative.

4. DISCUSSION

S. aureus is recognized worldwide as frequent cause of intramammary infections in dairy cows. The main reservoir of S. aureus seems to be the infected quarter, and transmission between cows usually occurs during milking. S. aureus produces a spectrum of extracellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism (Mounir et al., 2010).

Staphylococci aureus is non-capsulated and facultative anaerobic Gram-positive coccus which appear as grape–like clusters when viewed through microscope although pairs and short chain are seen frequently in fluid media. On cultivation it has a large, round, smooth, convex and lustrous golden-yellow colonies often with hemolysis when grown on blood agar plates. The golden appearance is the etymological root of the bacterium's name, aureus mean "golden" in Latin. It is non-spore forming non-motile. The organisms are found in suppurative process in man and animals (Ryan and Ray., 2004).

Results in this study revealed that staphylococci on Gram staining werenon-capsulated Gram-positive spherical cocci 0.8 – 1 µm in diameter in films from solid media grape-like clusters vary in size with some single or paired cocci in broth is small groups, pairs and short chains (less than 5 cocci in line). They are non-sporing and non-motile. These results are in agreement withCollee et al. (1989) and Koneman et al. (1992). In this study, Staphylococcus cultural characteristics on nutrient agar, white, yellow or orange water insoluble pigments were formed. And onmannitol salt agar,yellow or golden yellowwater insoluble pigments were formed.Alsothey were aerobic and facultative, liquefied gelatin and fermented a number of carbohydrates to acid. These results agreed withMerchant and Packer(1956) and Jahan et al.(2015).Staphylococcus cultural characteristics onBaired-Parker's agar media for 24-48 hours at 37

^{o}C, produced black, shiny, convex colonies with entire margins and clear zones surrounding the colonies with or without an opaque zone. These results agreed with that of Roberson et al. (1992) and Taylor and Francis (2010). The results of bacteriological examination of the clinical mastitis milk samples showed that the incidence of suspected staphylococci were 33isolates out of 117 (28.2%)examined milk samples. These results are nearly similar to those described by Jha et al. (1993) and Jha et al. (1994) who reported an incidence of 32.1 and 35.2%, respectively for staphylococcal species.The results of biochemical identification of the 33 suspected staphylococci revealed that 30.3 % were S. aureus. These results are nearly coincident with those obtained by Baudet and Chieze (1994) who isolated S. aureus at a percentage of 30% and Selem et al. (2002) at a percentage of 31.5%. The results of antibiotic susceptibility are in agreement with those of described by Mounir et al. (2010) who reported that danofloxacin, amoxicillin-clavulanic acid, sulbactam-ampicillin and Trimethoprim-sulphamethoxazolewere the most effective antibiotics for S. aureus strains susceptibility rates of S. aureus to these antibiotics were: 94% danofloxacin, 84% for amoxicillin-clavulanic acid, 82% for sulbactam-ampicillinand 75%for trimethoprim-sulphamethoxazole. However, high resistancerates were found to penicillin and streptomycin69%, oxytetracycline67%, cefoperazone andamoxicillin 59%and lincomycin 35% among S. aureus strains. The ability of S. aureus to adhere to extracellular matrix proteins is thought to be essential for the colonization and the establishment of infections (Salasia et al., 2004). S.aureus possesses various adhesion genes, including clfA, fnbA, andcan(El-Sayed et al., 2005).PCR analyses of clfA genes in the investigated 10 strains suggesting an important role of these elements in the pathogenicity of bovine mastitis. However, Brody et al.,(2008) found that some genes including clfA and S. aureus protein A gene spawere present in both antimicrobial resistant and susceptible isolates, statistical analysis showed there is a strong relationship with resistance patterns. Therefore, the determination of mechanism of antibiotic resistance patterns might provide important information towards specific control measures (Ferens et al., 1998). In this study most of the S. aureus strains were found to be resistant streptomycin, kanamycine, erythromycin, tetracycline, amoxicillin, cefoperazon, ampicillin and gentamycine at high rates. These high rates can be attributed to the random use of antibiotics dry period treatments, different treatment choices used in farms of ElBohera Governorate. All isolate included in this study exhibited a negative PCR reaction with the Integron class -1 gene. This result agreed with Xuet al., (2011) which showed that class 1 integron has been identified as a primary source of antimicrobial resistance genes in Gram-negative organisms. However, most available occurrence of integrons had been limited within Gram-negative microbes, very little is known for clinical Gram-positive bacteria (Nandiet al.,2004). Although all isolate was negative for class 1integronyet, this study started the investigation about class 1 integron in S. aureus from bovine mastitis milk in Egypt.

5. CONCLUSION

Data presented in this study showed that a broad distribution of identical or closely related S. aureus clones are responsible for the mastitis situation in El Bohera Governorate, Egypt. Due to S. aureus isolates from cows with bovine mastitis were found to differ in their gene patterns, genotypic characterization provided 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. Further studies aim to obtain more data about different species will be conducted indifferent regions in Egypt.

6. REFERENCES

Ahmed, A.M., Motoi, Y., Sato, M., Maruyama, A., Watanabe, H., Fukumoto, Y. and Shimamoto, T. 2007. Zoo animals as a reservoir of Gram-negative bacteria harboring integrons and antimicrobial resistance genes. Appl. Environ. Microbiol. 73: 6686 -6690.

APHA, American Public Health Association. 1984. Compendium of methods for the microbiological examination of foods. 2nd ed. American Public Health Association, Washington DC.

Bailey, W.R. and Scott, E.G. 1974. Diagnostic microbiology. A text book for the isolation and identification of pathogenic microorganism. The CV Moser Company, Saint Louis.

Baired-Parker, A.C. 1962. An important diagnostic and selective medium for isolating coagulase positive staphylococci. J. Appl. Bacteriol. 25: 12–19.

Baudet, H.M. and Chieze, C. 1994. Investigation into the nature and frequency of bacterial species isolated from subclinical mastitis at drying. Aff. Bulletin Mensnelde la SocieteVeterinair de France., 78 (3): 129–136.

Boerlin, P., Kuhnert, P., Hussy, D. and Schaellibaum, M. 2003. Methods for identification of S. aureus in cases of bovine mastitis. J. Clin. Microbiol. 41 (2): 767–777.

Brody, T., Yavatkar, A.S., Lin, Y., Ross, J., AKuzin, M.Kundu., Fann, Y. and Odenwald, W.F., 2008. Horizontal gene transfers link a human MRSA pathogen to contagious bovine mastitis bacteria. PLOS ONE, 3: 3074.

Clinical Laboratory Standards Institute. 2006. Performance standards for antimicrobial disk susceptibility tests; Approved standard-9th ed. CLSI document M2-A9. 26:1. Clinical Laboratory Standards Institute, Wayne, PA.

Collee, J.G., Fraser, A.G., Marmion, B.P. and Stilmmow, A. 1996. Practical medical microbiology. 14th Ed. Churchill, Livingstone. New York.

Cruickshank, R., Duguid, J.P., Marmion, B.P. and Swain, R.H.A. 1975. Medical Microbiology. Vol. II, the Practice of Medical Microbiology. 12th Ed., Churchill Livingstone, Edinburgh, London.

De Freitas Guimarães F., Nóbrega, D.B., Richini-Pereira, V.B., Marson, P.M., Figueiredo Pantoja, J.C. and Langoni, H. 2013. Enterotoxin genes in coagulase-negative and coagulase-positive staphylococci isolated from bovine milk. J. Dairy Sci. 96:2866-2872.

Delbes, C., Alomar, J., Chougui, N., Martinand, J.F. and Montel, M.C. 2006. Staphylococcus aureus growth and enterotoxin production during themanufacture of uncooked, semihard cheese fromcows raw milk. J. Food Prot., 69: 2161-2167.

El-Sayed, Alber, A., Lammler, J.C., Bonner, B., Huhn, A., Kaletaand, E.F. and Zschock, M. 2005. PCR based detection of genes encodingvirulencedeterminants inStaphylococcus aureus from birds.J . Vet. Med. B. Infect. Dis. Vet Public Health, 52: 38-44.

Ferens, W.A., Davis, W.C., Hamilton, M.J., Park, Y.H., Deobald, C.F., Fox, L. and Bohach, G. 1998. Activation of bovine lymphocyte subpopulations by staphylococenterotoxin C. Infect. Immun., 66: 573-580.

Gilbert, F.B., Fromageau, A., Gelineau, L. and Poutrel, B. 2006. Differentiation of bovine Staphylococcus aureus isolates by use ofpolymorphic tandem repeat typing. Vet. Microbiol., 117: 297-303.

Hugh, R. and Leifson, F. 1953. The taxonomic significance of fermentative versus oxidative metabolism of Carbohydrates by various Gram-negative bacteria. J. Bacteriol., 66: 24–26.

Jan Hudzicki and Kirby-Bauer. 2013. Disk Diffusion Susceptibility Test Protocol. http://www.microbelibrary.org/templates/beez/images/ml-hdr-ns.jpg

Jha, V.C., Thakur, P.P., Yadov, J.N. and Rail, B. 1993. Epidemiological investigation of subclinical bovine mastitis in the eastern hills of Nepals. Vet. Rev. Kathmadu., 8(2): 25–39.

Jha, V.C.; Thakur, R.P. and Yadov, J.N. (1994): Bacterial species isolated from clinical bovine mastitis and their antibiotic sensitivity patterns. Vet. Rev. Kathmandu., 9(1): 21–23.

Jean, F. and Macfaddin, F. 1976. Biochemical tests for identification of medical bacteria. The Williams and Wilkins Company, Baltimore. Md. 21202, USA.

Kalorey, D.R., Shanmugam, Y. Kurkure, N.V., Chousalkar, K.K. and Barbuddhe, S.B. 2007. PCR-based detection of genes encoding virulence determinants in Staphylococcus aureus from bovine mastitis cases. J. Vet Sci, 8:151-154.

Koneman, E. W., Allen, S.D., Janda, W.M., Schrechenberger, P.C. and Winn, W.C. 1992. colour Atlas and textbook of diagnostic microbiology (Fourth edition), 1979, 1983, 1988, 1992 by J. B. Lippincott Company.

Lee, J., Ha, J., Son, S.Y., and Han, K. 2012. Human genomic deletions generated by SVA- associated events. *Comparative Functional Genomics***2012**:807270.

Martineauf, P., Roy, P.M. and Bergeron, M.G. 1998. Speciesspecific and ubiquitous-DNA based assay for rapid identification of staphylococcus aureus. Clin. Microbiol.36:618-623.

Mason, W.J., Blevins, J.S., Beenken, K., Wibowo, N., Ojha, N. and Smeltzer, M.S. 2001. Multiplex PCR Protocol for the Diagnosis of Staphylococcal Infection. Journal of Clinical Microbiology, Vol. 39, No. 9, p. 3332–3338.

Merchant, J.A. and Packer, R.A. 1956. Veterinary Bacteriology and Virology. 5th Ed.

Mounir M. Salem-Bekhit, Muharram, M.M., Ibrahim M.Alhosiny. M. and Ehab S.Y. Hashim. 2010. Molecular Detection of Genes Encoding Virulence Determinants in Staphylococcus aureusStrains Isolated fromBovine Mastitis. Journalof Applied Sciences Research, 6(2): 121-128.

Mueena Jahan, Marzia Rahman, Shafiullah Parvej, M.d., Shah, M.d., Ziqrul Haq Chowdhury, Enamul Haque, M.d., Abdul Khaleque Talukder, M.d. and Sultan Ahmed. 2015. Isolation and characterization of Staphylococcus aureus from raw cow milk in Bangladesh. J. Adv. Vet. Anim. Res., 2(1): 49-5.

Sobhan Nandi, John J. Maurer, Charles Hofacre and Anne O. Summers. 2004.Gram-positive bacteria are a major reservoir of Class 1 antibiotic resistance integrons in poultry litter. PNAS May 4, vol. 101 no. 18 7119.

QIAamp® DNA Mini and Blood Mini Handbook.109024602/2015.

Quinn, P.J., Makey, B.K., Carter, M.E. and Carter, G.R. 1994. Textbook of Clinical Veterinary Microbiology. Elsevier, Mosby Ltd

Quinn, P.J., Makey, B.K., Carter, M.E., Donnelly, W.J. and Leonard, F.C. 2002.

Veterinary Microbiology and Microbial Diseases. Blackwell Science Ltd.

Roberson, J. R., Fox, L.K., Hancock, D.D. and Besser, T.E. 1992. Evaluation of methods for the differentiation of coagulase-positive staphylococci. J. Clin. Microbiol.30:3217–3219.

Ryan, K.J., Ray, C.G.,and Sherris, E.D.S. 2004. Medical Microbiology (4th ed.). McGraw Hill. ISBN 0-8385-8529-9.

Salasia, S.I.O., Khusnan, Z., Lammler, C. and Zschock, M. 2004. Comparative studies on pheno- and genotypic properties of Staphylococcus aureusisolated from bovine subclinical mastitis incentral Java in Indonesia and Hesse in Germany., J. Vet.Sci., 5: 103-109.

Sambrook, J., Fritscgh, E.F.and Mentiates. 1989. Molecular coloning. A laboratorymanual.Vol !., Cold spring Harbor Laboratotry press New York.

Selem, R.S., Rashed, A.M.O. and Fahmy, B.G.A. 2002. Mastitis pathogens: Attachments
related virulence features a whey protein markers and antibiotic efficacy in cows. Vet. Med. J. Giza, 50(3): 405–418.

Taverna, F., Negri, A., Piccinini, R., Zecconi, A., Nonnis, S., Ronchi, S. and Tedeschi,G. 2007. Characterisation of cell wall associated proteins ofa Staphylococcusaureus isolated from bovinemastitis case by a proteomic approach. Vet. Microbiol., 119: 240-247.

Taylor and Francis Group, LLC. 2010. International Standard Book Printed in the United States of America Number-13: 978-1-4398-0408-7.

Viana, D., Selva, L., Segura, P., Penades, J.R. and Corpa, J.M. 2007. Genotypic characterization of Staphylococcus aureus strains isolated from rabbit lesions. Vet. Microbiol., 121: 288-298.

Wada, M., Lkhagvadorj, E., Bian, L., Wang, C., Chiba, Y., Nagata, S., Shimizu, T., Yamashiro, Y., Asahara, T. and Nomoto, K. 2010. Quantitative reverse transcription-PCR assayfor the rapid detection of methicillin-resistant Staphylococcus aureus. Journal of Applied Microbiology 108 (2010) 779–788.

Xu, Z., Li, L., Shi, L. and Shirtliff, M.E. 2011. Class 1 integron in staphylococci. Mol. Biol. Rep.; 38(8): 5261–5279.