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Research Article
Livestock-Associated *Staphylococcus aureus* Pathogenicity with Regards to Resistance and Virulence Genomics and Accessory Gene Regulator Locus Proteomics

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Abstract

Background and Objective: Livestock-associated multidrug-resistant *Staphylococcus aureus* (LA-MRSA) pathovars with characteristic genetic profiles could lead to differences in antimicrobial responsiveness, reduced treatment susceptibility, however, increased pathogenicity. In this study the prevalence of some LA-MRSA pathovars with characteristics genomic and metabolomics profiles within 3 animal species in specific epidemiological settings in Egypt were investigated. **Materials and Methods:** The study included small-holders farmers from 10 localities samples collected during 2014- 2016. The focus was exclusively on individuals with untreated skin abscess (pigs), recurrent mastitis (cows), apparently healthy carriers (goats). The interrelationships were evaluated among novel isolates with regards to *agr* locus expression, antimicrobial resistance pattern, virulence factors genetic profiles and antimicrobial-induced time to clearance of MRSA bacteremia. A chi-square test had estimated the prevalence of each gene profile where differences are significant at $p<0.05$ (SPSS 19). **Results:** Six hundred MRSA field isolates molecularly classified by 16S rDNA amplification (241 bp), sequencing, then blastn to genbank data base were included. The virulence factors; clumping factors A protein (*clfA* = 638 bp), thermo-nuclease (*nuc* = 395 bp), toxic shock syndrome toxin (*tsst* = 326 bp) and exfoliative toxins (*etb* = 226 bp) genes, were determined. Moreover, the accessory gene regulator locus (*agr*) expression in relation to α and δ Hemolysis grad was estimated that ranged from 0- +4 in a strain's virulence-dependent manner. In addition, antimicrobial resistance genes profiles were determined to guide treatment trails; methicillin (*mecA* = 310 bp), vancomycin (*vanA* =1030 bp) and erythromycin (*ermC* = 295 bp). Finally, the antimicrobial-induced time to clearance of LA-MRSA bacteremia were compared between studied animal species. **Conclusion:** It was concluded that clearance of LA-MRSA was significantly dependent on the interactions of the virulence factors, antimicrobial resistance genes and the *agr* locus expression in relation to hemolysis grad. Hence, considering these future strains of unknown pathogenicity, they could reveal sub-acute, untreated, recurrent and/or food poising diseases.

Key words: LA-MRSA, mastitis, recurrent septicemia, antimicrobial resistance, virulence genes, agr locus

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

A wide spectrum of diseases including mastitis, recurrent septicemia, superficial skin endures and soft tissues invasions are provoked by *Staphylococcus aureus* species[1-5]. Strains isolated from camels, horses, dogs, cats, pigs, goats and other domestic and wild livestock were classified as human and non-human lineages of *S. aureus*[6-10]; livestock associated MRSA (LA)-MRSA), yet there is a scarcity within information about this particular pathogen in Egypt[1-5]. A comprehensive assortment of virulence exoenzymes are released encompassing; nucleases, proteases, lipases, hyaluronidase, deoxyribonuclease and collagenase with performance either alone or in concert, henceforth, the pathogenesis is sophisticated[11,12]. *Staphylococcus aureus* strains release a cascade of related pyrogenic toxins of the same genes cluster including staphylococcal enterotoxins (*ses*), toxic shock syndrome toxin (*tsst*), exfoliative toxins (*ets*) A and B, hemolysis (alpha, beta, gamma and delta) and Panton Valentine leukocidin (*pvl*)[9,11], therefore, detection of one of these genes usually indicates the presence of other enterotoxin genes[12-27]. TSST and ETS toxins provoke toxic shock syndrome and staphylococcal peeling skin syndrome, respectively[9,11]. Clumping factors A protein (*clfA*); biofilms formation enhancer, is the substantial protagonist in the asymptomatic infections [9,25-27]. The interruption of biofilms has been a focus of a huge form of research, which had utilized thermo-nuclease (*nuc*) as a biofilm dispersal agent[22-24]. *In vivo*, thermo-nuclease (*nuc*) has shown to efficiently influence colonization, prevent biofilm formation, clear existing biofilms and counter attack NETs entrapping [21, 24]. Two active nucleases (Nuc1 and Nuc2), encoded by different open reading frames are present in the *S. aureus* genome[18]. Nuclease activity showed a strong correlation with the *nuc*1 levels suggesting its being the major *S. aureus* nuclease[27]. Production of Nuc1 is conserved across methicillin susceptible *S. aureus* and MRSA strains and is therefore used as a unique marker to distinguish *S. aureus* from other staphylococcal species[1-5,12].

The quorum-sensing mechanism launched by the accessory gene regulator (*agr*) locus in *S. aureus* controls expression of the vast majority of virulence and housekeeping genes, where the increased *agr* expression increases secreted virulence factors and decreases cell-associated surface adhesins[28]. The *agr* locus is polymorphic, defining 4 alleles (I, II, III, IV) and consisted of the divergently transcribed P2 and P3 operons. The A-D genes of P2 operon are responsible for regulation of virulence remarks activation. The *agr* system, RNA III and the delta-hemolysin gene are the constituent of P3 operon. The bulk of the attenuated *agr* function *S. aureus* isolates was associated with reduced antimicrobial treatment success and MRSA prolonged bacteremia[29-31]. On the other hand, α, β, δ, hemolysin and PVL toxins are coded by *hla*, *hlb*, *hld*, *hlg* and *lukD-lukE* genes, respectively[12].

These enzymes/toxins synergistic effect, subsequently, leads the emergence of LA-MRSA lineage that acquired cumulative resistance and/or narrowed susceptibility to a few antibacterial agents, mostly the glycopeptide family of antibiotics; selective pressure[13-15]. Vancomycin was the drug of choice; nevertheless, slow bactericidal activity, poor tissue penetration and inactivity against bacterial virulence factors are its main disadvantages[16,17]. Moreover, vancomycin-resistant *S. aureus* isolates are being identified even in Egypt[1-5,18], where the Minimal Inhibitory Concentration (MIC) increased from 1-8 μg mL^{-1}, moreover, complications in therapy and treatment failures are the most dangerous prognosis[19,20]. This resistance was also noted with erythromycin, oxacillin, methicillin and other β-lactam antibiotics[1-5]. The genetic basis of LA-MRSA phenotypic profiles is unknown yet; the *in vivo* behavior could not be confirmed by the genetic profile[21]. Consequently, the reduced susceptibility to antimicrobials is based on selective pressure enforces gradual adaptive resistant due to complex mechanisms producing changes in cell wall biosynthesis, thickness, composition, intermediary metabolisms and cross-linking that generates designated antibiotics-resistant phenotypes of *S. aureus*[22-24]. Thus, additional treatment strategies are indispensable to improve clinical response, in addition, to reduce further antibiotic resistance expansion, preferably, those targeting virulence proteins directly thereby captivating *S. aureus* and rendering them more susceptible to host innate immune defenses[12].

The present study employed a set of well-characterized MRSA bacteremia isolates obtained from swine to evaluate potential interrelationships among novel collection of Egyptian field MRSA isolates from goats, cows and pigs with regards to *agr* locus expression, antimicrobial resistance pattern, virulence factors genetic profiles and antimicrobial-induced time to clearance of MRSA bacteremia. The research team focused exclusively on bacterial isolation positive individuals with untreated skin abscess (pigs), recurrent mastitis (cows), apparently healthy carriers (goats) to evaluate duration of bacteremia in settings where metastatic abscess

formation potentially confounding aspects play an integral role in microbiological and clinical outcomes of MRSA infections.

MATERIAL AND METHODS

Animals population and geographical scope of the study: The present study was carried on pigs (males, n = 80), goats (females, n = 50) and cattle (females, n = 20) with history of skin abscess and apparently healthy individuals during 2 years. Samples collected from 10 localities in Cairo, Giza and upper Egypt governorates from small holders farmers housing mixed populations of farm animals and poultry in the backyards of their houses during 2014 till 2016. Their age ranged 2-5 years considering the seasonal variations during the year. According to farmers' interview and case records, animals were fed for fattening mainly and medical care were given when clinical abnormalities are seen. The skin (all), udder and teats of (females) were examined by visual inspection and palpation for abnormalities before sampling. Skin abscess swabs, pharyngeal swabs and milk samples were collected from swine, caprine and bovine, respectively.

Clinical swabs: Skin and pharyngeal swabs were collected from swine and caprine, respectively, especially those individuals with untreated skin abscess (pigs) and in contact with apparently healthy species (goats).

California mastitis test: California Mastitis Test (CMT) was done on cattle before quarter-milk sampling; hence, it is not routinely applied within investigated localities. The results were read and evaluated with modifications according to Zecconi *et al.*[32] and Guliye *et al.*[33].

Collection of milk samples: After stripping about 10 mL of milk, duplicate quarter's milk samples were collected under strict standardized procedures. The samples were transported to the laboratory at 4°C. Then, specimens were incubated for 2 h at 37° C for bacterial enrichment before undertaking the analysis while those for SCC evaluation were kept at 4°C[34].

Milk somatic cell counts: Milk samples specified for SCC evaluation were analyzed within 24 h from collection. They were pre-warmed at 37°C for 10 min then automatically measured using SOMA-COUNT 150 (Bentley, USA). The log10 (SCC) values had classified udders status into 3 categories; normal udder recorded SCC below 4×10^5 cells mL^{-1}, while apparently normal subclinical mastitic udders possessed SCC above 4×10^5 cells mL^{-1}, finally above 1×10^6 representing the mastitic udders[33].

Bacteriological survey on pathogens causing recurrent bacteremia

Standard procedures for Staphylococcus aureus isolation: Equal volumes of 10 µL of each sample (milk and rehydrated swabs) were simultaneously plated on Nutrient agar, Manitol salt agar, 5% defibrinated sheep blood agar (Biolife Laboratories, Milano, Italy) then incubated aerobically at 37°C[35]. Identification of *S. aureus* isolates using API system; bacterial colonies were sub cultured onto Columbia blood agar at 37°C for 18-24 h, then single young culture was inoculated into API staph medium to make a homogeneous bacterial suspension with a turbidity equivalent to McFarland tube No. 0.5 and this suspension was used immediately after preparation. Identification was obtained with the numerical profile on the result sheet, 20 tests of API strip. The biochemical tests were selected from Kloos and Schleifer scheme[35].

***Staphylococcus aureus* planktonic culture conditions:** *Staphylococcus aureus* obtained isolates on solid plates were grown in 3 mL brain heart infusion broth at 37°C with agitation[36]. After overnight incubation, the optical density at 560 nm was determined; mid-exponential (OD_{560} = 1.0) and stationary (OD_{560} = 3.5) growth phases. Equal number of cells (1.5-2 mL of each culture) was harvested by centrifugation at 12000 rpm/10 min at 4°C to be used later on during molecular techniques.

Bacterial isolates and reference strains: *Staphylococcus aureus* ATCC 25923 was used as control positive strain. In addition to, 12 local swine isolates that have previously been identified as *S. aureus clfA*$^+$; two isolates each was genetically; [*mecA*$^+$ *ermC*$^+$ *vanA*$^+$], [*mecA*$^+$ *ermC*$^-$ *vanA*$^+$] and [*mecA*$^+$ *ermC*$^-$ *vanA*$^-$], three isolates were [*mecA*$^+$ *ermC*$^+$ *vanA*$^-$], one isolate each was [*mecA*$^-$ *ermC*$^-$ *vanA*$^-$], [*mecA*$^-$ *ermC*$^-$ *vanA*$^+$] and [*mecA*$^-$ *ermC*$^+$ *vanA*$^-$][5].

Standard procedures for identification of field isolates of *Staphylococcus* spp.: The characterized bacterial isolates from collected samples were categorized as clinically relevant or a contaminant by clinical and laboratory criteria. For epidemiological records, the criteria included clinical signs, physical examination findings and body temperature at the time of the sampling, leucocyte and differential cell counts,

Table 1: List of oligonucleotides primers used in this study

Target	Gene	5`-Sequences-3`	Fragment bp	References
PCR internal control	16S rRNA	F: GGAGGAAGGTGGGGATGACG	241	Martineau *et al.*[40]
		R: ATGGTGTGACGGGCGGTGTG		
Species-specific classification	*clfA*	F: GCAAAATCCAGCACAACAGGAAACGA	638	Mason *et al.*[41]
		R: CTTGATCTCCAGCCATAATTGGTGG		
Virulence factors determinants	*tsst*	F: ACCCCTGTTCCCTTATCATC	326	Mehrotra *et al.*[42]
		R: TTTTCAGTATTTGTAACGCC		
	etb	F: ACAAGCAAAAGAATACAGCG	226	
		R: GTTTTTGGCTGCTTCTCTTG		
	nuc	F: ATATGTATGGCAATCGTTTCAAT	395	Gao *et al.*[45]
		R: GTAAATGCACTTGCTTCAGGAC		
Antibiotic resistance determinants	*mecA*	F: GTAGAAATGACTGAACGTCCGATAA	310	McClure *et al.*[43]
		R: CCAATTCCACATTGTTTCGGTCTAA		
	vanA	F: CATGAATAGAATAAAAGTTGCAATA	1030	Kariyama *et al.*[44]
		R: CCCCTTTAACGCTAATACGATCAA		
	ermC	F: ATCTTTGAAATCGGCTCAGG	295	Schlegelova *et al.*[20]
		R: CAAACCCGTATTCCACGATT		
qRT-PCR internal control	*gyrB*	F: CGCAGGCGATTTTACCATTA	-	Seidl *et al.*[47]
		R: GCTTTCGCTAGATCAAAGTCG		
agr-RNAIII expression	RNAIII	F: GCCATCCCAACTTAATAACCA	-	
		R: TGTTGTTTACGATAGCTTACATGC		

SCC results, number of positive cultures out of the total number performed and response to treatment[37]. A semi quantitative δ-hemolysin functional assay was performed to assess and score *agr* locus function (from 0 to+ 4)[38].

Molecular characterization of *Staphylococcus* isolates to species and subspecies levels

Bacterial DNA isolation: For DNA extraction pellet of each isolate was suspended in 180 µL Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8, Sigma Aldrich) containing 100 µg mL^{-1} of lysostaphin (Sigma Aldrich) and boiled for 5 min. then centrifugation at 12000 rpm/10 min to remove debris. The extraction mixture containing 20% SDS (Sigma Aldrich) and 100 µg mL^{-1} proteinase K (Qiagen) in 400 µL TE buffer (Sigma Aldrich) was added to 40 µL of each bacterial aliquot and then incubated for 1 h at 37°C[39]. The extraction was done by phenol: Chloroform: Isoamyl alcohol; 25:24:1 mixture (Sigma Aldrich)[39]. Purity and integrity of extracted genomic DNA were investigated by NanoDrop 2000c (Thermo Scientific). The working DNA concentration was adjusted[39-47] to 100 ng $µL^{-1}$. Primers used during the present study were synthesized by Metabion International AG, Semmelweissstr, Germany. All of the PCR assays were performed in 25 µL total volumes in PTC-100™ Thermal Cycler (MJ Research Inc., USA).

Internal quality control for PCR assays: To confirm that PCR inhibition was absent and to reduce the formation of nonspecific extension products, a semi-qualitative internal control to verify the efficiencies of the DNA isolation and the PCR assays was applied. Primer used derived from highly conserved regions of the bacterial 16S rRNA gene (Table 1)[40]. The PCR thermal cycling was as the following: (3 min at 96°C and then 30 cycles of 1 sec at 95°C, 30 sec at 55°C and 7 min at 72°C for the denaturation, annealing and extension steps, respectively, then one cycle of extension step 10 min at 72°C) finally cooling done to 4°C[40]. A reagent blank (containing all the components of the reaction mixture with MillQ water instead of genomic DNA) was used as control negative. Pure fully characterized colonies of *S. aureus* ATCC 25923 were utilized during the optimization of the PCR.

Amplification of *S. aureus* species-specific *clf*A gene: Oligonucleotide primers set (Table 1) was designed with reference to previous publications[41] where an ubiquitous 638bp fragment within *clf*A gene was amplified from genomes of clinical Staphylococcus isolates of swine, caprine and bovine origin obtained during the present study. Each PCR mix included; 3 µL of DNA (100 ng $µL^{-1}$), 50 pM $µL^{-1}$ of each primer, 10 mM dNTP, 25 mM $MgCl_2$, 5 U $reaction^{-1}$ Taq DNA polymerase and nuclease free water to complete the volume of each reaction (Qiagen). The PCR reactions were subjected to one cycle of initial denaturation at 94°C for 5 min. Then 35 cycles included; 94°C for 1min, 55°C for 1 min and 72°C for 1 min, followed by 10 min extension at 72°C, finally the reactions were cooled down to 4°C[41].

Molecular identification of *S. aureus* virulence remarks and antimicrobial-resistance genetic profiles: The *tsst*, *etb*, *nuc mecA*, *vanA* and *ermC* oligonucleotide primers sets (Table 1) were designed with reference to previous

publications where an ubiquitous 326, 226, 395, 310, 1030 and 295 bp fragments within each gene were amplified[20,42-45], respectively, from genomes of clinical Staphylococcus isolates of swine, caprine and bovine origin obtained during the present study. The PCRs cycling profiles were carried out according to previous publications[20,42-45].

Sequencing of PCRs products: Each amplicon was purified for sequencing using the ExoSAP-IT PCR Product Cleanup kit (Affymetrix, USA) according to the manufacturer's instructions. Sequencing reactions were performed with the ABI PRISM®BigDyedye™ terminator cycle sequencing kit with AmpliTaq® DNA polymerase on an MJ Research PTC-225 Peltier Thermal Cycler (Applied Biosystems, USA) as described by the manufacturer. Each sequencing reaction was repeated at least 3 times in both directions before being accepted for analysis. Then sequences of each PCR product was aligned with homologous genbank records (http://www.ncbi.nlm.nih.gov) by multiple sequence alignment using the Clustal®W program[46].

Measurement of S. *aureus agr* expression profiles: The *agr*-RNAIII transcripts from post exponential phase were assayed by means of quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR). The RNA was isolated with the Ambion TRI Reagent solution (Invitrogen) according to the manufacturer's protocol. Pellets were lysed with lysing matrix tubes (MP Biomedicals) and the Bead Beater FastPrep Fp120 at 6000 rpm for 40 sec (Bio 101, Savant). RNA extracts were treated with Ambion Turbo DNA-free Kit (Invitrogen), according to the manufacturer's protocol. Complementary DNA was synthesized from 750 ng of RNA by using an RT enzyme mix (Invitrogen) and 250 ng of random primers (Invitrogen). The qRT-PCR was carried using the SYBR Green ER qPCR Super Mix and a real-time PCR detection system (iQ5, Bio-Rad Laboratories). Reaction mixtures were prepared using 250 nmol L^{-1} RNAIII-bis-F and -R primers pair (Table 1) resulted in earlier detection signals as evaluated by the comparison of Ct values[47,48]. Distilled water served as a negative control. Relative expression levels were determined by comparison to the level of *gyrB* gene expression (internal control, Table 1) in the same cDNA preparations[47,48]. Analysis of the melting curve of specific PCR products was performed by slowly raising the temperature from 60-95°C by means of regular fluorescence measurements, which should be distinguished from primer dimmers (dissociation temperature <74°C)[47,48].

Antibiotics minimal inhibitory concentrations and time to bacteremia clearance: The vancomycin, erythromycin and clindamycin MICs were determined using broth microdilution methods[49] and E-test under conditions suggested by the manufacturer (AB Biodisk). In vitro antibiotics killing assays were performed in duplicate with use of a starting inoculum of 1×10^6 CFU mL^{-1} in Mueller-Hinton broth containing each/plate of vancomycin, erythromycin and clindamycin where the concentration was selected to represent target through serum levels recommended for treatment of bacteremic *S. aureus* infection[29,50]. Bactericidal activity was defined as a reduction of $\geq1\times10^3$ CFU mL^{-1}, compared with the starting inoculum. Statistical validation correlated between time to clearance of MRSA bacteremia (days) and ≥1 of the preceding genotypic and phenotypic profiles of each isolates from included animal species. Time to bacteremia clearance was defined as the time, in days, from the initiation of antimicrobial therapy until the first day with negative blood cultures after the last positive culture. To eliminate investigator bias, all outcomes and clinical information were determined and documented before *in vitro* testing and all *in vitro* testing was performed by investigators blinded to the clinical status and outcome data[29,50].

Data analysis and statistics: A chi-square test was used to compare the prevalence of each gene profile among *S. aureus* isolates between categories (SPSS 19). Differences between the prevalence rates were considered significant when $p<0.05$[51].

RESULTS

Health impression and bacteremia clearance time from CMT/SCC analysis of dairy bovines/caprines and swine skin abscesses ion: The visual inspection of the females udders; goats (n = 50) and cattle (n = 20), did not display systemic symptoms except for those with clinical mastitis. In addition, the milk samples were obviously normal, especially in goats. After analysis of both CMT and SCC scores, 15 mastitic (10 cows, 5 goats), 35 subclinical mastitic apparently healthy (7 cows, 28 goats) and 20 healthy (3 cows, 17 goats) females were classified, respectively. For SCC controls, 2 normal bacteriologically negative milk samples of healthy females of both species were randomly selected. The clinical mastitis cases were completely recovered after 10 days of treatment with antibiotics; the 1st day with negative blood cultures after the last positive culture (Table 2). The subclinical apparently healthy individuals' suffered intermittent untreated

Table 2: Resistance determinants and virulence genes profile with regards to *agr* locus expression in *S. aureus* species clinical isolates obtained during present study

			Hemolysis[a]		
Animal species	Specimens	*S. aureus subspecies aureus*	δ	α	Bacteremia clearance time[b]
Bovine (F, n = 20)	Milk (n = 15) Blood (n = 20)	*clfA*$^{+}$, *tsst*$^{+}$, *etb*$^{+}$, *nuc*$^{+}$, *mecA*$^{+}$, *vanA*$^{+}$, *ermC*$^{+}$	++++	++	After 10 days from treatment of mastitis
		clfA^{+}, *tsst*$^{+}$, *etb*$^{-}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{+}$, *ermC*$^{-}$	+++	+	Intermittent bacteremia, apparently healthy, subclinical mastitis
		clfA^{+}, *tsst*$^{+}$, *etb*$^{+}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{+}$, *ermC*$^{+}$			
		clfA^{+}, *tsst*, *etb*$^{+}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{-}$	++	+/-	
		clfA^{+}, *tsst*$^{+}$, *etb*$^{-}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{+}$, *ermC*$^{-}$			
		clfA^{+}, *tsst*, *etb*$^{-}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{-}$			
		clfA^{-}, *tsst*$^{-}$, *etb*$^{-}$, *nuc*, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{-}$	+	++	
Caprine (F, n = 50)	Milk (n = 33) Pharynxes (n = 50)	*clfA*$^{+}$, *tsst*$^{+}$, *etb*$^{+}$, *nuc*$^{+}$, *mecA*$^{+}$, *vanA*$^{+}$, *ermC*$^{+}$	++	+	Recurrent bacteremia, apparently healthy, subclinical mastitis
		clfA^{+}, *tsst*$^{+}$, *etb*$^{+}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{+}$, *ermC*$^{-}$	+	++	
		clfA^{+}, *tsst*, *etb*$^{-}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{-}$			
		clfA^{+}, *tsst*, *etb*$^{-}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{-}$			
		clfA^{-}, *tsst*$^{-}$, *etb*$^{-}$, *nuc*, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{-}$	+/-	++	
Swine (M, n = 80)	Skin abscess (n = 60)	*clfA*$^{+}$, *tsst*$^{+}$, *etb*$^{+}$, *nuc*$^{+}$, *mecA*$^{+}$, *vanA*$^{+}$, *ermC*$^{+}$	+++	++	Recurrent bacteremia, untreated skin abscess
		clfA^{+}, *tsst*$^{+}$, *etb*$^{+}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{-}$			
		clfA^{+}, *tsst*, *etb*$^{+}$, *nuc*$^{+}$, *mecA*$^{+}$, *vanA*$^{-}$, *ermC*$^{-}$			
		clfA^{+}, *tsst*, *etb*$^{-}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{+}$			
		clfA^{+}, *tsst*, *etb*$^{+}$, *nuc*$^{+}$, *mecA*$^{+}$, *vanA*$^{-}$, *ermC*$^{-}$			
		clfA^{+}, *tsst*, *etb*$^{-}$, *nuc*$^{+}$, *mecA*$^{+}$, *vanA*$^{+}$, *ermC*$^{+}$	+	++	
		clfA^{+}, *tsst*$^{+}$, *etb*$^{+}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{-}$			
		clfA^{+}, *tsst*$^{+}$, *etb*$^{-}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{+}$			
		clfA^{+}, *tsst*, *etb*$^{-}$, *nuc*$^{+}$, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{-}$			
		clfA^{-}, *tsst*$^{-}$, *etb*$^{-}$, *nuc*, *mecA*$^{-}$, *vanA*$^{-}$, *ermC*$^{-}$	+/-	-	

[a]Results of α and δ-haemolysin toxins are listed semi-quantitatively in four categories[48]. -: Not present, +/-: Borderline, +: Present, strong activity in three grades (++, +++, ++++), [b]Response to antibiotic treatments, subsequently, bacterial clearance time was previously determined how to be estimated[48], where positive response $\geq$5 log10 CFU mL^{-1} and/or $\geq$3 log10 CFU g^{-1} reductions per sample, while recurrent bacteremia usually recorded $\leq$1.5 log10 CFU mL^{-1} reduction per sample due to treatment

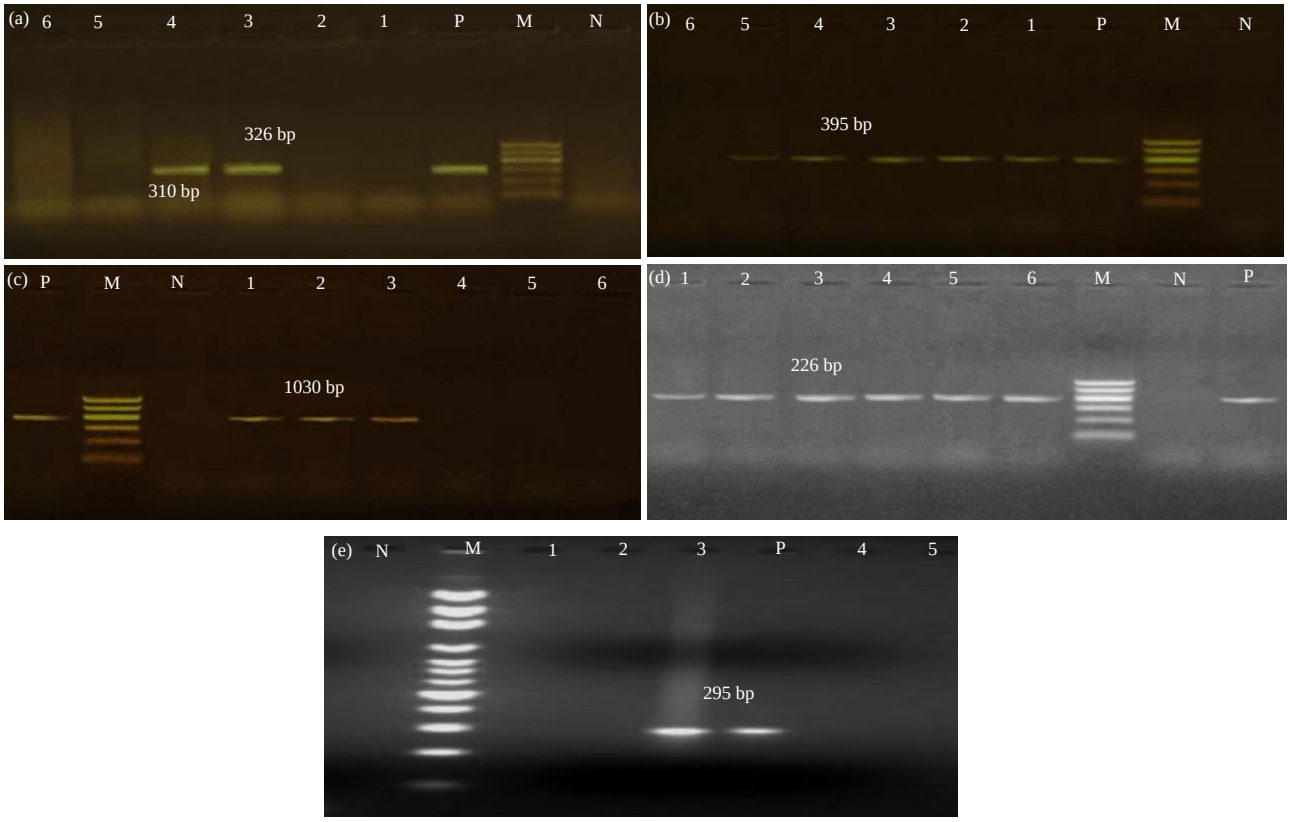

Fig. 1(a-e): Antimicrobial-resistance and virulence remarks genetic profiles of some *S. aureus subsp aureus* clinical isolates obtained during present study. The *mecA*, *tst*, *nuc*, *vanA*, *etb* and *ermC* ubiquitous fragments with molecular sizes; (a)310 bp and 326 bp, (b) 395 bp, (c) 1030 bp, (d) 226 bp, (e) 295 bp

bacteremia that weakly responded to treatment (Table 2). In case of swine, recurrent bacteremia and/or untreated skin abscess were the main obvious sings which were difficult to respond to antibiotics.

Prevalence of *S. aureus* species clinical infections within investigated animal species: The examined individuals revealed 600 bacterial isolates on stroked agar plates from subclinical/mastitic as well as healthy females, pharyngeal swabs and skin abscess. Two clinical forms could be recognized: Single (30%) and mixed (40%) bacterial infections; noting that 30% of the samples with no bacterial growth which indicated the need for other pathogens specific media. The phenotypic characterizations of *S. aureus* are previously described. The obtained colonies indicated obvious alterations including changes in cell morphology and in the thickness which is in accordance with previous publications[47,50].

Molecular identification of *S. aureus* Subspecies aureus based on *clfA*, virulence remarks and antimicrobial-resistance genes annotation: According to conventional PCR procedures, the detection limitation of DNA/assay adjusted to be 10 ng of genomic DNA, which was obtained from 10^6 CFU of each field isolate. Negative results were obtained from blanks (Fig. 1); in addition, expected amplifications were obtained from the reference DNA in each PCR trials as well as from internal controls (Fig. 1). Positive results with bands possessed the specified molecular sizes for *clfA*, *tsst*, *etb*, *nuc*, *mecA*, *vanA* and *ermC* genes specific for *S. aureus* spp. (Table 2), respectively, were visible in designated isolates lanes within agarose stained with ethidum bromide (Fig. 1).

Sequences of the amplified genes were compared with those available in GenBank using gapped BLASTN software. Identification to the species and subspecies levels was obtained by the fragments with sequences identities >97% [52].

BLASTN analysis of the aligned sequences of the isolates showed 99-96% identity with the similar genes sequence of *S. aureus subsp. aureus* strain M013 (accession No. CP003166). BLASTN analysis of the aligned sequences of the isolates showed 81-95% identity with the similar genes sequence of *S. aureus subsp. aureus* other then strain M013 (accession No. CP003166). Phylogenetic analyses grouped present

isolates into branches with *S. aureus subsp aureus* strains; NCCP14562 (CP013955), Mu3 (AP009324), M1 (HF937103), Mu50 (BA000017), Bmb9393 (CP005288), 6850 (CP006706), CN1 (CP003979), SA957 (CP003603), SA40 (CP003604), Z172 (CP006838), USA300ISMMS1 (CP007176), NRS 100 (CP007539), UAS391_USA300 (CP007690), XN108 (CP007447), SA268 (CP006630), VC40 (CP003033), in addition to, 84 extra *S. aureus Subspecies aureus* in one clade separated from other species and subspecies. According to incidence of infectious bacterial isolates, the research team considered *S. aureus Subspecies aureus* is the primary causative agent in recurrent bacteremia, mastitis and skin abscess induction. The obtained genes sequences were recorded in genbank.

Relationship between MRSA *in vitro* susceptibility, *agr* locus function, virulence remarks and antimicrobial-resistance genes and bacteremia clearance time: Antimicrobials MIC was significantly related to both *agr* function and clinical case prior antibiotics use ($p<0.001$, data not shown). The MRSA with attenuated *agr* function (δ-hemolysin score, 0-2) had significantly higher Antimicrobials MICs and increased morbidity and mortality rates ($p<0.001$, data not shown). Treatment recipes were isolates' genetic profile dependent (Table 2). It was recorded that the species mixed raring conditions maintained the reservoirs for recurrent bacteremia (goats and pigs), failure of treatment in some animal species (certain cows, goats and pigs) and increased phenotypic variability of cultured colonies which indicated pronounced genetic variability on both structural and functional levels (Fig. 1 and Table 2).

The *agr* locus expression could be quantitated in variant *S. aureus* cultures in direct relationship to RNAIII gene expression. Analyzing the qRT-PCR amplification plot (the melting curve) of RNAIII gene; subsequently, *agr* locus expression, could indicate variant Cycle Threshold (Ct) ranged: 23.64, 26.81, 30.34, 33.34 and 36.77 for δ-hemolysin scores 4+, 3+, 2+, 1+ and +/-, respectively. These results are positive reactions indicative of ranged amounts of the targets RNAIII copy number/bacterial culture/sample, subsequently, high to weak and/or zero δ-hemolysin particle number/*S. aureus* culture (Table 2).

DISCUSSION

Multidrug-resistant *S. aureus* have been discovered in a diverse range of hosts, suggesting that they are more common than had been suspected[1-5]. Believing that ecological characteristics of animal housing influence the epidemiology and clinical aspects of diseases triggered the choice of the animals population included in the present study. It is obviously noted that mixed housing conditions of animal species specially in backyards maintained the *in vivo* reservoirs for recurrent bacteremia (goats and pigs), failure of treatment in other individuals (mastitic cows, goats and pigs)[6-11,53] and increased phenotypic variability of isolated bacterial pathovars which indicated pronounced genetic variability on both structural and functional levels (Fig. 1 and Table 2)[37,39,48,53]. These ecological considerations in MRSA transmission could be the explanation for negative results of PCRs in other individuals under similar housing conditions; subsequently, governorates being MRSA-free (data not shown) despite the recorded animal trading between studied locations[3,11]. Such notes highlighted the cryptic role of rodent's populations which are commonly in contact with swine along the two sides of river Nile, since, we still do not have a comprehensive point of view on how MRSA bacteremia (pathogenesis) are maintained symptomless within reservoir populations or how they move horizontally between species[1-5,7-10]. Thus better understanding of these dynamic processes should be the next target to achieve by detailed studies of representatives from the different animals and in contact human groups, collected from the designated governorates.

Fortunately, the obtained results did not represent a conflict with, however, supported previous reports proved the existence of MRSA in Egypt in different animal species[1-10]; cattle, goats and swine. In addition to, *in vivo* positive correlation with virulence and reduced antimicrobials responsiveness were defined[1-5]. It is well understood that there is a differential response to the treatment of infection based on interactions of host, pathogen and antimicrobial pharmaceutical group[48,53]. Therefore, the heterogeneous nature of susceptibility to glycopeptides, applied in the treatment, has resulted from discordance between microbiological and clinical behavior of MRSA among obtained clinical infections, subsequently, heterogeneous antimicrobial responsiveness and *agr* expression (Table 2)[15-18]. While appearing straightforward, antibiotic susceptibility as measured *in vitro* via the determination of the MIC may be complicated by microbial phenotypes too subtle to be detected by standard laboratory methods [3,13-20]. Subsequently, response to antibiotic treatments was positively correlated to bacterial clearance time; which was mainly estimated depending on reduction of bacterial count *in vivo*[48]; where positive response had $\geq$5 log10 CFU mL^{-1} and/or $\geq$3 log10 CFU g^{-1} reductions per sample, while recurrent

bacteremia usually recorded ≤1.5 log10 CFU mL^{-1} reduction per sample due to treatment.

The topology of phylogenies inferred by analyses of amplified MRSA-sequences of certain virulence remarks and antimicrobial-resistance genes and those counterparts in genbank enabled the detection of pathovar that are 96-99% similarity to records of strain from human and nonhuman origins[12-14,21]. However, obtained genetic clusters illustrated a degree of relationship in between Egyptian amplicons than to other *S. aureus* species records despite the hosts' differences. The fact that these local isolates are characterized by limited and specific geographical distribution, furthermore, the wide distance and lack of contact between the targeted locations in this study with those surveyed previously confirmed the inter- and intra-*S. aureus* subspecies similarities differences in between isolates recorded in the present study[1-5]. Furthermore, the existence of mixed infections detected by PCR may be a violent situation proposing horizontal gene transfer between isolates. This has important implications for the evolution of recurrent bacteremia as genes can sweep through different genetic backgrounds of *S. aureus* pathovars thereby altering bacterial pathogenicity and transmission capability[37,47-48,54-55]. On the other hand, the neglected role of rodents habituating the grooves within backyards and maintaining these pathovars seems disastrous, because they can help to elucidate the mechanisms of pathogenicity, transmission and virulence of MRSA[39, 54-55].

Staphylococcus aureus is internalized (haematogenous spread) by a variety of host's nonprofessional phagocytes; pathogenesis of infectivity involved invasion and damage of endothelial cells was proved previously, thus it serves as a '*in vivo* biomarker' for certain aspects of immunological interactions maintaining clinical bacteremia[48,55]. Hence, *S. aureus* avoids host immune resistances as well as the bactericidal effects of many antimicrobial agents, fostering persistent and/or relapsing infections[48,55]. In the present study, interestingly, the extent of internalization estimated by bacterial density and/or count on isolation was neither related to a pathovar's ability to invade tissues, protease/nucleases production nor toxin gene transcription but to functionality of *agr* locus for maximal tissues damage induction and hemolysis. Upon *S. aureus* entrance into the bloodstream, endocytosis is immediately triggered as much as invasion and proliferation is induced at the initial infection sites to develop persistent clinical infections. Essentially, *S. aureus* intracellular elaboration of exoenzymes/toxins; precisely *nuc* gene and *agr* locus, induces host cell lysis, facilitating haematogenous spread to other target organs consequently dissemination to distant sites. However, adherence to and subsequent invasion of endothelial cells are required but not sufficient for *S. aureus* to induce complete cellular damage; only altered and/or immature functionality (cytokines) is fundamental for bacterial virulence, cellular damage and antimicrobial responsiveness. First, the extent of endothelial cellular damage by different MRSA strains predicted their virulence in terms of achievable bacterial densities in the target tissues. Those MRSA strains which induced less cellular damage were also significantly less $p<0.05$ virulent as compared with MRSA strains that caused greater damage. Second, there was a significant inverse $p<0.05$ correlation between MRSA-induced cellular damage and antibiotic therapeutic responses in the study. Thus; with one exception (strain 300-246) internationally recorded, higher blood cells damage correlated with worse response to antibiotic therapy. This differential *in vivo* clinical outcome among studied pathovars was not linked to several standard *in vitro* susceptibility technologies; including MICs, killing kinetics, tolerance or population analyses profiles. Moreover, commercially available panels for the identification of staphylococci; based on functional genomics differences by metabolic pathways solely, do not allow the reliable typing distinction of *S. aureus* to subspecies level. Therefore, they are not optimal for the diagnosis of LA-MRSA infections. Hence, the development of a rapid and reliable method for the identification of these pathogens that can also be applied for detection directly from clinical specimens is needed[37,55]. Besides, further characterization using polyphasic strategy combining genotypic and phenotypic profiles are mandatory, to facilitate recording these local isolates within collection of the WHO and/or ATCC under reference number.

CONCLUSION

LA-MRSA pathogenicity was dependent on metabolomics profiles that are the outcomes of functional genomic profiles with diverse construction between isolates. In addition, the microbial genetic contents imposes a selective pressure for emerging new isolates of unknown pathogenicity and transmission capability for both human and animals communities; rather than nonpathogenic. The discordance noted between microbiological and clinical behavior of local MRSA isolates triggers heterogeneous host susceptibility to the antimicrobial treatments applied during the study. Therefore, clinical care should be positively correlated to bacterial count *in vitro*, simultaneous with, bacterial clearance time *in vivo*. The LA-MRSA isolates studied could be associated with sub-acute, untreated recurrent or food poisoning diseases, which is imposing negative economic impact on livestock industry.

SIGNIFICANCE STATEMENTS

This study discovers the interactive effect of the virulence factors, simultaneously, the accessory gene regulator locus (*agr*) expression in relation to hemolysis grad and antimicrobial resistance genes profiles on the antimicrobial-induced time to clearance of LA-MRSA bacteremia in between individuals with spectrum of diseases in three animal species. This study will help the researcher to uncover the critical area of horizontal gene transfer between LA-MRSA isolates thereby altering bacterial pathogenicity and transmission capability. Thus, a new spotlight on the neglected role of rodents habituating the grooves within backyards maintaining these pathovars to elucidate the mechanisms of pathogenicity, transmission and virulence of LA-MRSA is added to practitioners and veterinarians.

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