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PVL and QACA Genes of Biofilm Positive Methicillin Resistant *Staphylococcus Aureus* (MRSA) Isolated From Human

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Abstract

This study was carried out to determine the existent of Panton-Valentine leukocidin (*pvl*) and quaternary ammonium compounds (*qacA*) genes among Methicillin-resistant *Staphylococcus* (S.) *aureus* (MRSA) producing biofilm. The total 150 patient nasal samples from various areas in Baghdad province, Iraq were collected during the period from October 2020 to April 2021. Samples were streaked on mannitol salt agar (MSA) and incubated at 37°C for 24 hours then, sub-cultured on blood agar. The isolates identified by phynotipic method including colony morphology, Gram stain and biochemical test and analysis profile Vitek® 2, antibacterial resistance and MRSA was determined by disc diffusion analysis. The biofilm synthesis was detected by Congo red agar method. The genotypic analysis of MRSA isolates using deoxyribonucleic acid (DNA) extraction to detect the *pvl* (359bp) and *qacA* (394bp) genes by conventional PCR assay. Of the 150 nasal swabs 28 (18.6%) were positive to MRSA, The majority of MRSA isolates (89.28%) were biofilm positive MRSA. The isolates revealed high rate of multidrug resistance 82.14%. Genotypic analysis by PCR showed that the *PVL* and *qacA* genes were detected in 60.71 and 78.12% of human isolates respectively, the biofilm positive isolates of human were carried the *PVL* and *qacA* genes 48%, and 88% respectively. The detection of *pvl* and qacA genes in biofilm positive MRSA isolates in this study suggests the need for the application of surveillance programs.

Keywords: Panton-Valentine leukocidin, quaternary ammonium compounds, PCR, Oxacillin, Iraq

Introduction

Staphylococcus aureus produces an extracellular matrix of proteins, carbohydrates, and extracellular DNA on its own which envelope the cells within a viscous matrix that facilitates survival in extreme or hostile environments cause chronic inflammation due to its resistant to treatment by formation biofilms on indwelling medical devices, (Moormeier *et al.*, 2017).

The pathogenicity of *S. aureus* is caused by the action of numerous potential virulence factor including enzymes surface compound toxins such as PVL, which leading to serious infections including many oral diseases such as periodontitis, peri-implantitis, oral mucositis, and even dental caries, toxic shock syndrome, septicemia, endocarditis necrotizing pneumonia (hemorrhagic), food poisoning, soft tissue infections as well as bone and skin infections (Reddy, *et al* 2017). *PVL* is a potent cytotoxin and an important virulence factor of *S. aureus*, causing tissue necrosis and

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selectively disrupts leukocyte membranes, thus, leading to enhanced virulence of *S. aureus*, in addition, the agents involved in the virulence of *S. aureus*, its role as an efficient opportunistic pathogen (Karmakar *et al.*, 2016).

Biocides based on the *qacA* gene are widely used for disinfection and preventing the infection and the transmission of bacteria in a hospital, extensive and inadequate use of these disinfectants for disinfection could lead to a selective pressure for the survival of bacterial strains with *qacA* resistant genes, which creating a potentially serious problem of infection control in hospitals and clinical settings (Morgan, 2008). Nowadays MRSA is one of the more frequent germs found in biofilm linked infections partially result from the reality that they are both human skin and mucosae commensal, but also common source serious infections with high morbidity, mortality, and healthcare associated costs (Nasution *et al.*, 2018; Gatta and Al-Graibawi, 2021). The capability of bacteria to produce a biofilm is regarded an important virulent property in its pathogenesis (Oniciuc *et al*., 2016). Therefore, it is necessary to develop new drugs and seek effective methods that would affect the bacteria in biofilms. The aim of the current study was to detect the PVL and qacA genes in Biofilm Positive MRSA Isolated from Human.

Materials and Methods

Sampling

One hundred and fifty (150) human nasal swabs were collected from different locations in Baghdad province, Iraq during the period from October 2020 to April 2021. Swabs were inserted in sterile tubes containing transport media and transported by ice cooler pox to the bacteriology laboratory, Zoonotic diseases unit, College of Veterinary Medicine, University of Baghdad. This study was approved by The Committee of Ethics Research of the Zoonotic Diseases Unit, College of Veterinary Medicine, University of Baghdad, Ministry of High Education and Scientific Research-Iraq (No. 1177/ 23-9-2020).

Isolation and Identification of MRSA

The isolation and identification of *S. aureus* were performed using the standard microbiological technique, primary isolation was done by streaking the swabs on mannitol salt agar (MSA), the suspected colony was re-grown on blood agar plates and incubated at 37°C for 24 hrs, identification of the isolates was carried out according to colony characterization, Gram staining, conventional biochemical analysis including hemolysis, catalase, coagulase, oxidase, DNase and mannitol fermentation tests (Markey *et al*., 2014). In addition, to analysis profile Vitek® 2 compact system (BioMérieux, France).

Detection of MRSA

Phenotypic detection of MRSA strains was performed by methicillin disk diffusion assay, the inhibitory zones around these discs were measured by millimeter (mm) unit by using a metric ruler, the isolates were classified as susceptible, intermediate and resistant to antibiotic the isolates showed resistant to methicillin were regarded as MRSA, (CLSI, 2020).

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Detection of biofilm

Twenty eight of MRSA were tested for Biofilm synthesis was detected by colony morphology on Congo red agar (CRA) plates as described by Freeman *et al.*, (1989).

PCR amplification

To extract genomic DNA the bacteria were streaked on nutrient agar, a colony was selected and inoculated in 4 ml of nutrient broth, 1 ml of the broth was transferred into a 1-5 ml tube for centrifugation for 20 min at 10,000 rpm, the supernatant was discarded and the cell pellet was used for extraction and purification of DNA using genomic DNA Kit (Geneaid, Thailand) according to the instructions of company. Concentrations and purity of extracted DNA was determined by Nanodrop spectrophotometer (Thermo, USA). Gel electrophoresis was used to detect DNA bands stained with red safe dye and visualized under UV light as described by Sambrook and Russell (2001). For DNA extraction 1g agarose was used. To generate PCR products 2g agarose was added to 100 ml 1XTBE buffer, boiled and cooled to 50°C For polymerization, 2µl of Red Safe dye was poured and left 30 min at room temperature. Gels poured into chambers then covered with 1 X TBE buffer. Then 10 µl samples were mixed with 5 µl dye buffer and loaded into wells with gel. DNA products were screened for the presence of *PVL* and *qacA* genes by PCR (Table 1), with specific primers (Bioneer, Korea) and PCR mixture of 20 µl with 2 µl premix of *Taq* DNA polymerase, MgCl2, dNTPs, KCl, stabilizer tracking dye and tris-HCl, 1 µl of each primer (final concentration was 10 picomole /µl) and 2 µl of DNA template then the volume was completed with sterile distilled water (Ibrahim and Al-Mathkhury, 2018). The *PVL* and *qacA* primers sequences used in this study as described in table (1).

Table (1): Types of primers used in PCR assay

Code	5`-3`	Product length (bp)
pvl-F	GTGCGCTGGTTGCTTAACAT	359
pvl-R	CATAAAATGCGCGCGGCGATGA	
qacA-F	CAATTGCACCCGGATTAGCG	394
*qac*A-R	ACAGCGCCCACTACAGATTC	

These primers that used (from Alpha DNA company, Canada) which were provide as lyophilized form and were dissolved in sterile deionize D.W to gave a final concentration 100 Pico mole /µl according to the recommendation of provider and then store in a deep freezer until use.

Amplification reaction

Table (2): Conditions of polymerase chain reactions

Gene	Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Final Extension
Pvl	94ºC / 7 min.	35	94ºC / 45 min	58ºC / 45 sec	72 ºC / 45 sec	72 ºC / 45 sec
qasA	94ºC / 7 min.	35	94ºC / 45 min	58 ºC / 45 sec	72 ºC / 45 sec	72 ºC / 45 sec

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Before electrophoresis, PCR products for each well were loaded with 5 µl sample. DNA ladders 100 bp and 1kb (KAPA Biosystem, USA) were run concurrently with each electrophoretic run to detect size of amplified PCR product. Electrophoresis was performed at 70V for 1.5 hour for extracting genomic DNA and PCR products, then DNA bands visualized by UV trans illuminator (Scope-21/Japanand photographed by using a digital camera. The amplified DNA fragments were visualized by UV light illumination by staining with ethidium bromide. The PCR assay was performed in the College of Veterinary Medicine/ University of Baghdad.

Results

Isolation and detection

Out of 150 nasal swabs 28 (18.6%) were positive to MRSA, the colonies of *S. aureus* isolates appeared on mannitol salt agar as round, smooth, and fermenting as shown in Figures 1.

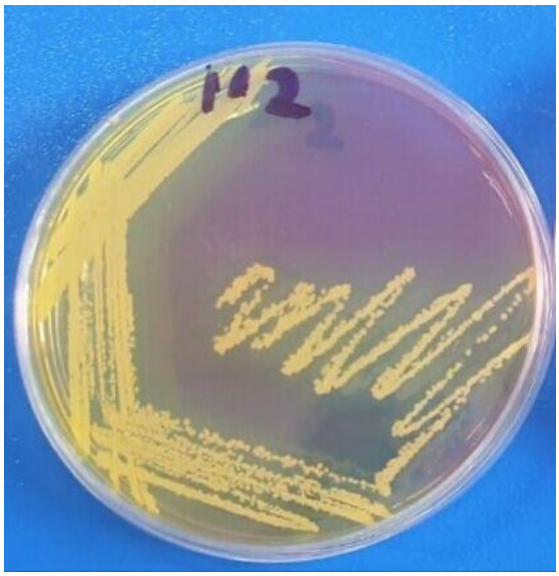

Figure (1): Growth of *Staphylococcus aureus* on mannitol salt agar

The isolates appeared as Gram positive, cocci, single cell pairs and grape-like clusters. The isolates were positive for catalase, gelatinase and DNase, coagulase tests and hemolysis (Table 3).

Table (3): Biochemical reactions for *Staphylococcus aureus*

Test	Result
Catalase	+
Oxidase	-
DNAse	+
Coagulase	+
Gelatin liquefaction	+
Hemolysis	+

Biofilm detection

Results of colony morphology of MRSA on CRA plates showed that 25 isolates (89.28%) formed black colonies (biofilm positive) (Figure 2).

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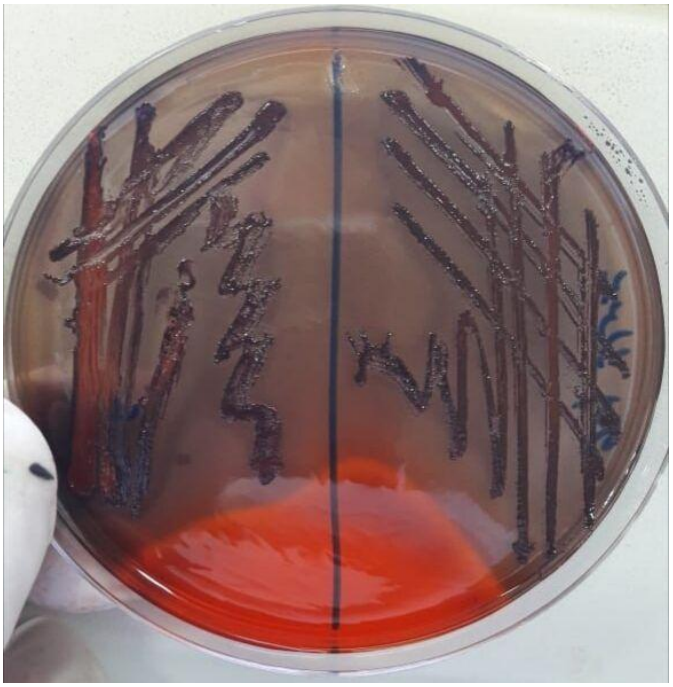

Figure (2): Biofilm detection by Congo red agar method, showing *Staphylococus asureus* positive (black colonies) Congo-red binding

Antimicrobial susceptibility testing

The MRSA isolates showed high rate of multidrug resistance 82.14%, with high resistance to penicillin and methicillin (100%), azithromycin (85.71%), fusidic acid (75%) and oxacillin (71.42%). However, it was sensitive to Bacitracin (100%), Norfloxacin (96.42%), Gentamycin and Clindamycin (85.71%), Vancomycin (71.42%), (Figure 3).

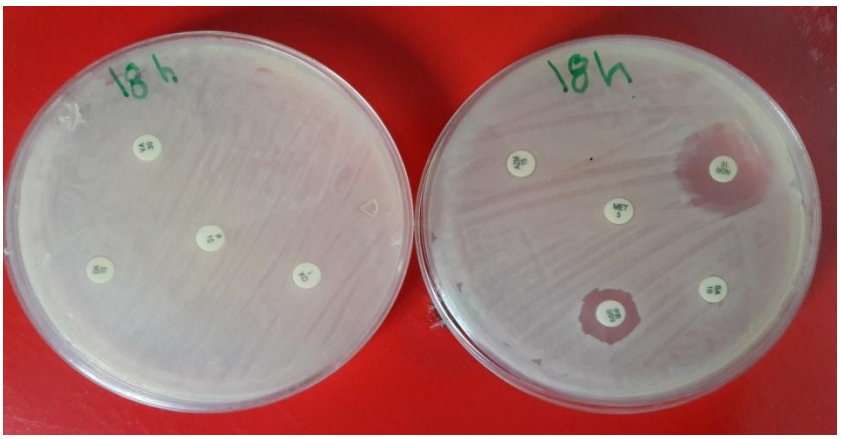

Figure (3): Antimicrobial resistant disc diffusion test of *S. aureus* isolates

Polymeras chain reaction results

Extraction of genomic DNA of *S. aureus*

The genomic DNA was well extracted from 28 human, were imagined by electrophoresis showed a good qualification of extracted genomic DNA (Figure 4). The concentration and purity of extracted DNA ranged between (1.8-2) as determined by using Nanodrop spectrophotometer.

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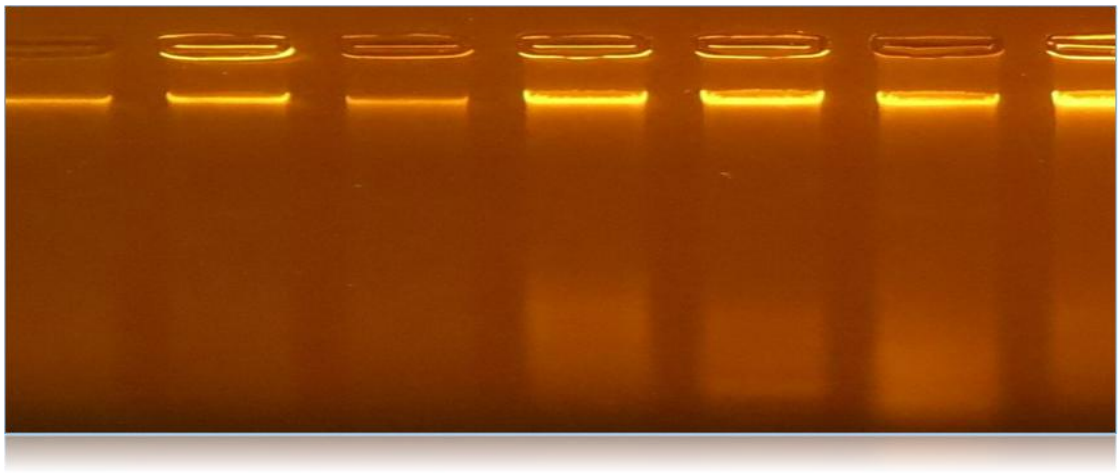

Figure (4): Genomic DNA of *Staphylococcus aureus* on agarose gel electrophoresis (1% agarose gel run at 90V, for 30 min) with ethidium bromide stain, loading template DNA after extraction from all the isolates

Detection of *PVL* and *qacA* genes by conventional PCR

The PCR analysis to detect *PVL* and *qacA* genes in the human isolates showed that the *PVL* and *qacA* genes were detected in 60.71 and 78.12% respectively (Figures 5, 6).

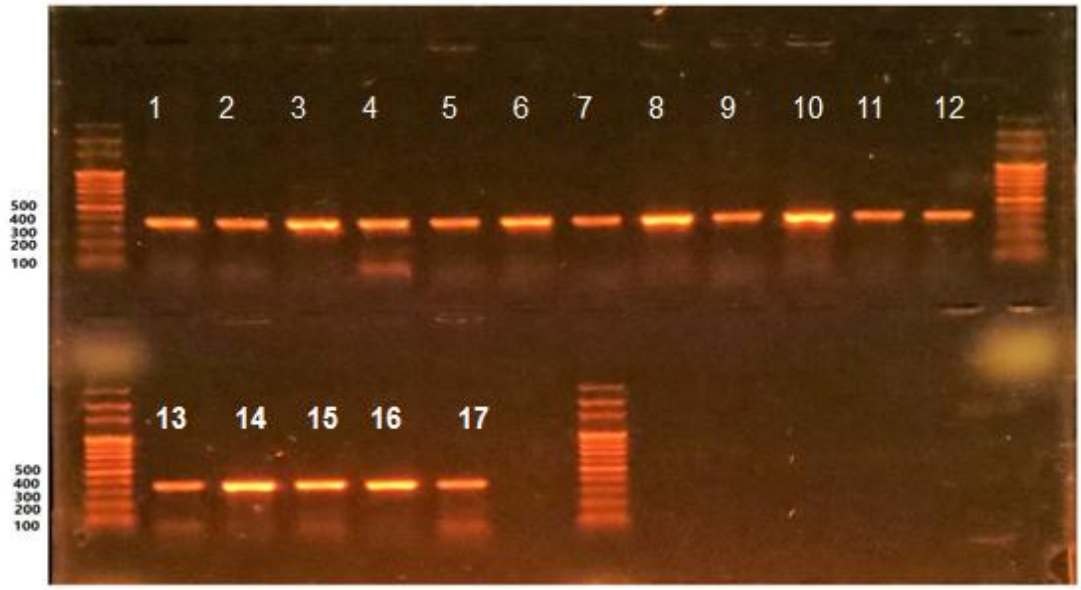

Figure (5): PCR product analysis of PVL gene (359pb) in *S. aureus* isolates on agarose gel electrophoresis. Where M: marker (100 -2000) bp, Lane 1-17: All the 17 positive isolates from human samples at PCR product

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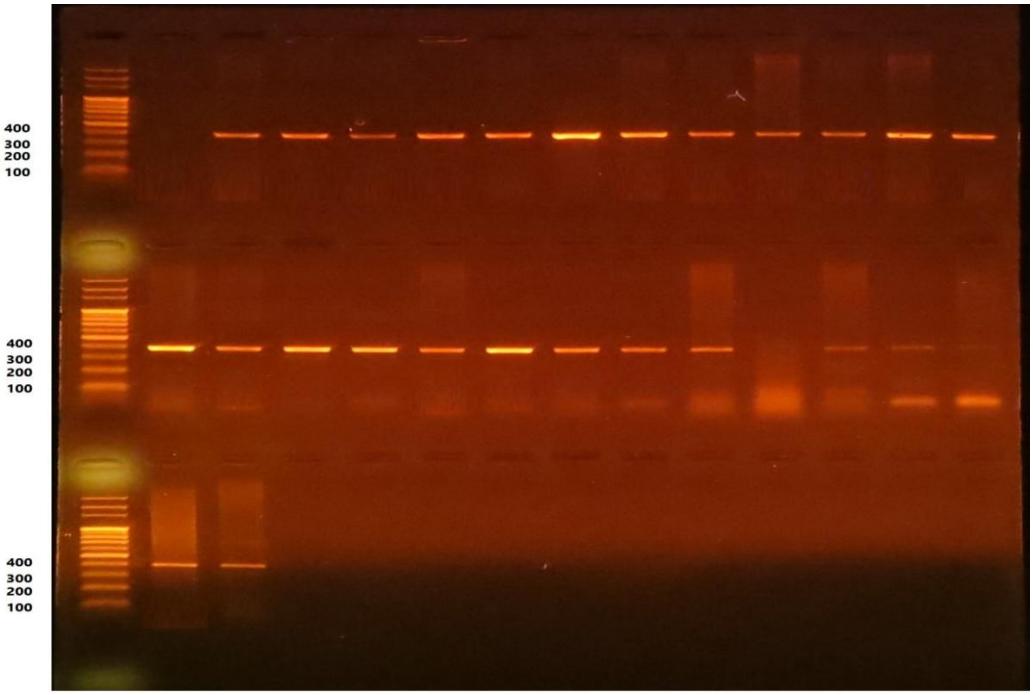

Figure (6): Product analysis of qacA gene (394pb) in *S. aureus* isolates on agarose gel electrophoresis. Where M: marker (2000 -100bp) 25 positive isolates from human samples at PCR product.

The majority of MRSA isolates were biofilm producers (89.28%), in these isolates*qacA* and *PVL* genes are correlated with biofilm production 88% and 48% respectively(Table 4)

Table (4): Association of biofilm positive MRSA on Congo red agar plates with *PVL* and *qacA* gene

Biofilm	*PVL*	*qacA*
Positive isolates 25/28 (89.28%)	**12/25 (48%)**	**22/25(88%)**

Discussion

Our cultural results (colony morphology, Gram staining and biochemical tests) demonstrated similar phenotypic characters for *S. aureus* as reported in previous studies (Markey *et. al*., 2014) *S. aureus* has long been considered as one of the main human pathogen, it is associated with nosocomial and community-acquired outbreaks worldwide (Morgan, 2008). The isolation rate in the current study was 18.6%, In dissimilarity, Lafi (2019) reported 42% incidence of human isolates in Baghdad governorate.

The growth of our isolates on Congo red media exhibited its ability to produce a slime layer, similar result was reported previously (Achek, 2020) the Congo red agar was used in the detection of the slime (biofilm) production from *S. aureus* isolated from human and animals (Horiuk *et al*., 2019). Early identification of biofilm forming *S. aureus* in clinical isolates could be an important method in control and prevention of the infections (Horiuk *et a*l., 2020).

Our *S. aureus* isolates were highly resistant 100% to methicillin, 71.42% to Oxacillin. The improper use of antimicrobial drugs poses great hazards to both animal and human health due to the emergence of antimicrobial resistance (Okocha *et al*., 2018).

The current results revealed that the isolates harboring 60.71% *pvl* gene in human, our finding, was higher than those reported by Ibrahim and Al-Mathkhury (2018) where the detection of this gene was 10% only. It is a potent cytotoxin and an important virulence factor of *Staphylococcus aureus*, causing tissue necrosis and selectively disrupts leukocyte membranes, thus leading to enhanced virulence. *Pvl-carrying S. aureus* strains can cause serious skin and soft tissue infections and life-threatening invasive diseases like necrotizing fasciitis (Miller *et al.,* 2005). This gene is present in the majority of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates studied (Lina *et al.,* 1999), and is the cause of necrotic lesions involving the skin or mucosa, including necrotic hemorrhagic pneumonia (McGrath *et al.,* 2008).

The results of *qacA* gene revealed that the isolates harboring 78.12% *qacA* gene in human, this study higher than that reported by Susanne *et al.* (2001) who detect the rate of 63% and disagree with Stefańska *et al.* (2002) who recorded that the isolate harboring 45% *qacA*. The rate of harboring *qacA* in clinical MRSA isolates varied significantly between different areas. The biocide genes with the most effect on chlorhexidine MIC in *S. aureus* isolates was *qacA*, followed by *qacB*, but *qacC* and different types of norA did not have any effect on chlorhexidine susceptibility (Morgan, 2017). Quaternary ammonium compounds (*QACs*) have a long-known application as antiseptics and disinfectants applied in various industries such as pharmaceutical, agricultural and food industry, given the alarming number of *QACs* resistant bacteria, there is an urgent need to develop new *QACs* with broad-spectrum antimicrobial activities and low tendency to trigger bacterial resistance (Odžak, 2020). Previously, Noguchi *et al.* (1992) were the first to analyze the distribution of the three antiseptic resistance genes in parallel.

Conclusion

The detection of *pvl* and qacA genes in biofilm positive MRSA isolates in this study suggests the need for the application of surveillance programs, coordinating the collection of data from the community, hospital, and livestock environments.

Acknowledgment

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