

Characterization and Identification of Methicillin-resistant *Staphylococcus aureus* (MRSA) Producing Biofilm: Impacts of Garlic Extract and *Lactobacillus Biosurfactants*

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The current study was intended to characterize and specify local MRSA isolates based on its phenotypic and genotypic features. Moreover, the anti-biofilm impact of garlic and *Lactobacillus bio surfactants* against *S. aureus* was examined. A total of 130 samples and isolates were taken from four sheep farms. Ninety samples were nasal, 10 were lung tissues and the remaining 30 were raw sheep meat from local grocery stores. All isolates were examined for the presence of *S. aureus* by routine and known microbiological tests. Oxacillin and cefoxitin disc diffusion test were done and findings were confirmed by PCR technology for *S. aureus* specific genes. Lastly, garlic water extract (gWE) and *Lactobacillus biosurfactants* were examined for their anti-biofilm activity utilizing a microtitre plate test. Fifteen isolates out of 75 *S. aureus* were specified MRSA. Concerning the PCR results, the 16SrRNA, the *nuc* and the *mec* Agenes were detected in 75, and 15 isolates, respectively. Moreover, *nuc* gene was present in all MRSA isolates (100%). The biofilms formation by *S. aureus* MRSA was repressed significantly by garlic water extract (gWE)and was dose dependently effect. Same repression was reported by *Lactobacilli biosurfactants*in dose dependent manner. The prevalence of *S. aureus* MRSA was confirmed and *S. aureus* biofilm activity was repressed by gWE and *Lactobacilli biosurfactants*.

Keywords: *S. aureus*, MRSA, Adhesion molecules, Congo red, Garlic Extract, PCR, Anti-biofilm activity.

Staphylococcus aureus (*S. aureus*) is a typical pathogen, causing a great variety of infections on skin¹. Treatment of *S. aureus* infections with antibiotics is frequently less powerful to insufficient because of the development of antibiotic-resistance strains, Methicillin-resistant *S. aureus* (MRSA)². The virulence of *S. aureus* pathogen relies upon the generation of several factors. There were 24 anchored cell wall proteins that expressed by *S. aureus*. These proteins promote *S. aureus* to cling to extracellular materials, influencing on the invasion of non-phagocytic cells and interference with innate immunity³.

MRSA was isolated and known by followings, hospital related MRSA (HA-MRSA), community-related MRSA (CA-MRSA) and livestock-associated MRSA (LA-MRSA)⁴. Nowadays, MRSA strains were isolated from different foods sources, such as poultry, pork, beef, milk and vegetables, concluded that foods may act as source of contamination⁵. *S. aureus* biofilms more significant virulence factor than any other strains. The development of biofilms on medical devices is a major issue in hospitals, as they can turn into a wellspring of disease⁶. *S. aureus* hinder the first line of defense of in the body known as immune system by building a wide variety of active peptides⁷.

Garlic (*Allium sativum* L.) contains active antibacterial substance called allicin, diallyl disulfide and alipin, which is powerful against numerous pathogenic microbes^{8,9}. S-allyl-L-cysteine sulfoxide (alliin) is a garlic organo-sulfuring reagent with no odor. The allinase enzyme, which is the cysteine sulfoxidase, turns alliin into allicin when garlic is cut. The strong smell, antioxidant and antibacterial functions of garlic are attributed to allicin¹⁰. The main biological, biochemical and antioxidant active substance of fresh garlic is named allicin. Probiotic bacteria, for example, *Lactobacillus fermentum* and *Lactobacillus plantarum*, found to repress *S. aureus* biofilm formation, however the mechanism is not well known. *S. aureus* genes *icaA* and *icaD* expression is engaged with the synthesis of the biofilm matrix¹¹.

The Current study was planned to isolate, characterize and identify local MRSA isolates with regards to genotypic and phenotypic features. Moreover, the activity of garlic water

extract (gWE) and *Lactobacillus biosurfactants* against biofilm formation by MRSA isolates were examined.

MATERIALS AND METHODS

Specimen collection and identification

A total of 130 samples were isolated and collected from four sheep farms and local grocery stores in Turabah governorate, Taif, Saudi Arabia. The collected samples were divided as following, 90 out of 130 samples were nasal swabs, 10 were lung tissues from recently dead sheep suffering from respiratory symptoms and 30 were raw sheep meat collected from local grocery stores. Collection of samples were ranged from October 2017 till April 2018. All samples obtained from live and dead sheep were obtained by veterinarians the guide lines of Taif University, Saudi Arabia. The collected nasal swabs and lung tissue samples were sub-cultured on tryptic soy broth (Difco) and then incubated for 24 hrs at 37 °C and were maintained on trypticase soy agar, (Difco). The meat samples (raw sheep) were taken and kept at 4 °C prior to investigation. 25 grams of meat samples were homogenized with buffered peptone water (225 ml) and 1:10 serial dilution was done. 0.1 ml of diluted samples were streaked on Baired-Parker (BP) agar (Oxiod) supplemented with egg yolk tellurite emulsion (Oxiod). The plates were then incubated for 24 hrs at 37 °C¹². Isolates were primarily identified by Gram stain, Catalase, Culturing on mannitol salt agar, coagulase test¹³ and molecular PCR technology.

After the confirmation of *S. aureus* from previous routine microbiological methods, isolates were identified for MRSA by *cefoxitin* and *oxacillin* disc diffusion test according to NCCLS guidelines, 2002¹⁴. Next, All resistant isolates to oxacillin (1 µg) and cefoxitin (30 µg) discs were confirmed by PCR technology for MRSA.

DNA extraction of MRSA and Triplex PCR identification

A triplex PCR technology was used to identify and discriminate the *S. aureus* from other *staphylococcal* species and MRSA isolates genotypically¹⁵. Then 500 µl of the broth was centrifuged for 5 minutes at 2000 rpm. For the pellet, 500 µl of DEPC-treated water (DNase-RNase free) was added and flushed in a vortex.

After boiling in water bath at 100°C for 10 minutes. The rubes were centrifuged at 10000 rpm for 5 min, the supernatant contain bacterial DNA was taken and used as a template for PCR.

PCR (triplex) was used to identify *S. aureus* from other *Staphylococci* species and to characterize MRSA genotypically. To detect *Staphylococcus spp.*, 16S rRNA gene was used. To detect *S. aureus* specific gene, *nuc* gene was used. The *mecA* expression as a methicillin resistance specific gene was used. The sequence of sense and antisense primers for each gene was used (**Table 1**). Exactly 5 ml of the rapid extracted DNA were taken as a template in a 25 µL PCR mixture. The PCR reaction was consisted of 5µL of cDNA, 2 µL of 10 pM of each primer, and 12.5 µL master mix for PCR (Promega Corp, Madison, WI, USA). The volume was adjusted to 25 µL with sterile deionized water. PCR reaction was performed in thermal cycler machine (Bio-Rad T100™). The primers were synthesized from Macrogen Co, Seoul Korea. The DNA amplification was performed as follows: 94°C for 4 minutes of initial denaturation; 30 cycles of 94°C for 50seconds, 58°C for 50 seconds and 72°C for 90 seconds; and a final extension at 72°C for 7 minutes. Amplified genes were loaded in 1.5% agarose gel (stained with ethidium bromide). The 756-bp (16S rRNA), 310-bp (*mecA*) and 279-bp (*nuc*) amplified DNA fragments were separated by electrophoresis in agarose gel and visualized under UV-light using gel documentation system.

Congo red Agar method for determination of biofilm production (CRA)

To detect biofilm production from MRSA isolates, a specific medium. The medium was composed of BHI; 37 gms/L, sucrose; 50 gms/L, agar agar; 10 gms/L, and congo red stain; 0.8 gms/L was used. Congo red medium was prepared and autoclaved. Then the MRSA isolates were

inoculated in plates with the medium and incubated for 24 and 48 hrs at 37°C¹⁶. Black (intense black) were considered strong positive. While dark colonies (black colonies) considered intermediate biofilm producers. Pink colonies mean weak biofilm producers, while the non-biofilm producers strains remain red with smooth appearance. The experiment was carried out three times¹⁷.

Microtitre plate test (MTP) for quantification of slime production of MRSA

The quantification of biofilm formation of isolated MRSA and typical positive on CRA was done as described before¹⁸, with little modifications. Isolates of MRSA were subcultured on TSB supplemented with 0.25% glucose overnight at 37°C with shaking. About 100µl were added to microtitre plate (96-well) contains 100µl of fresh TSB supplemented with 0.25% glucose. The plate was incubated for 24hrs at 37°C. Then, plates carefully washed by PB(PH 7.4) three times to remove none adherent cell. Then plates were dried in the air and maintained in inverted position before staining. Crystal violet (0.4%) was used to stain the adherent cells with a volume of 100µl per well for 120 seconds and then left to be air-dried and washed by sterile distilled water three times. Finally, Optical density (OD) was measured using spectrophotometer (Smart Spec[™] Plus- Bio- Rad- USA) at 570nm. The slime produced from isolates were classified into 4 categories based on obtained OD, strong slime formers ($OD_{570} \geq 3.0$), moderately slime formers ($OD_{570} \geq 1.5- 2.0$), weak slime formers ($OD_{570} \geq 0.5-1.0$) and none slime formers were ($OD_{570} \leq 0.5$).

Preparation of garlic water extract (gWE)

Garlic was purchased from local markets in Turabah, Saudi Arabia. Buds were cleaned thoroughly, then rinsed with tap water and left for drying for 24hrs at room temperature. Fifty grams of garlic was crushed in a sterile grinder

Table 1. Triplex PCR for identified MRSA genes

| Gene name | Direction (5'-3') | Gene sequence |
|----------------------|--------------------------------|--|
| 16 S rRNA (756-bp) | 16S rRNA-F 16S rRNA-R | AACTCTGTTATTAGGGAAGAACA CCACCTTCTCCGGTTTGTCCACC |
| <i>nuc</i> (279-bp) | <i>nuc-F</i> <i>nuc-R</i> | GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC |
| <i>mecA</i> (310 bp) | <i>mecA-F</i> <i>mecA-R</i> | GTAGAAATGACGAACGTCCGATA CCAATTCCACATTGTTTCGGTCTAA |

to obtain fine powder. Then 100 ml of sterile pure water were added to the powder and was left for overnight, filtration was done using Whatman paper No1. Finally, the extract was concentrated using shaking incubator (Model: TH2-300- SN 170824647- China) at 37°C for 6days. The obtained concentrated garlic extract was kept in deep freezer till use¹⁹.

Isolation of Lactobacillus bio surfactants (LAB) from Yoghurt

LAB was isolated from yoghurt purchased from local markets in Turabah, KSA. Briefly; one gram of yoghurt and 9 mL sterile saline water were

mixed. Then serially diluted and immediately plated on LB agar(Difco, Bacton, USA), which is selective for *Lactobacillus spp.* The plates were kept and incubated anaerobically, at 37 °C for 48 hrs. Catalase-negative, gram-positive and rod-shaped *bacilli* were considered LAB positive, further confirmation was made by culturing of previous obtained colonies on MRSA agar²⁰.

Anti-biofilm Activity

The anti-biofilm activity was explained before²¹ with some modifications. All isolates of MRSA were sub-cultured overnight with fresh sterile tryptic soy broth (TSB) supplemented

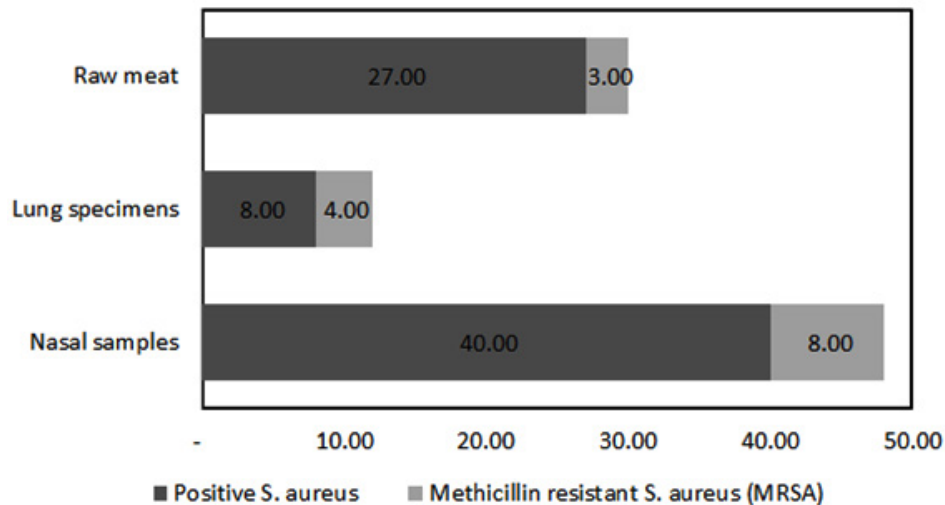


Fig. 1. Agar disc diffusion test of isolated *S. aureus*. The highest percent of MRSA was in nasal samples, then lung specimens and raw meat samples by 8, 4, and 3 respectively

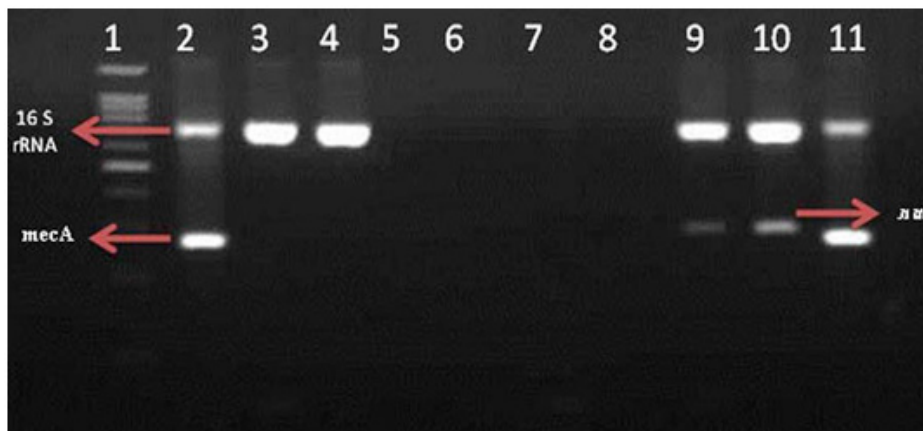


Fig. 2. Triplex-PCR of *S. aureus* and determination of methicillin resistance samples. Lane 1: DNA Marker (100 bp); lane 2: (nasal swab) positive *mecA*; Lanes 3,4 nasal swab containing 16S rRNA. Lanes 5-8: negative *nuc* and *mecA*. Lanes 9 and 10: lung samples are positive 16s rRNA, and *nuc*, while lane 11: meat sample, positive *mecA* and 16S rRNA

with 0.25% (w/v) glucose. Then, 100 µl of cultures of each isolate was transferred to 96-well microtitre plates. A volume of 100 µL of different concentration of garlic extract (12.5, 25, 50 and 100mg/ml) and *LAB biosurfactants*(50 and 100mg/ml) were added to each well except positive and negative control wells. Wells were incubated at 37 °C for 48 hrs, then gently washed twice with sterile PBS (PH 7.4)²². Thereafter, the biofilm were fixed using 200 µL methanol for 10 min, then stained with 0.1% crystal violet for about 10 min and rinsed 3times with distilled water. The adherent cells were dissolved using 200 µL of 33% acetic acid, OD was measured using spectrophotometer (Smart Spectm Plus- Bio- Rad-USA) at 570nm²⁰. Each experiment was repeated three times. Positive control contains bacterial isolates without

any treatment but negative controls contains only tryptic soy broth with 0.5% (w/v) glucose. The current results were expressed in biomass formation inhibition percentage²³.

Statistical analysis

Data are calculated and expressed as means ± standard error for values of MRSA isolates. All data analyzed using ANOVA (analysis of variance) by Bonferroni test for SPSS software version 11.5 for Windows (SPSS, IBM, Chicago, IL, USA). All values less than P < 0.05 we considered statistically significant.

RESULTS

Isolated *S. aureus* from nasal swabs, lung tissues of dead sheep and raw sheep meat were tested using agar disc diffusion method to examine the MRSA distribution (Figure 1). The obtained results revealed that 15 isolates (8, nasal; 4, lung and 3, raw sheep meat) were positive *S. aureus* MRSA specific species.

Triplex PCR was used to discriminate *S. aureus* from other *S. species*. Triplex PCR targeted the 16S rRNA, the *nuc* and the *mec* A genes to be specific genes for *Staphylococci*, *S. aureus* and *S. aureus* specific MRSA, respectively. Detected DNA fragments were with size of 756, 279 and 310 bp, for the 16S rRNA, the *mecA* and the *nuc* genes,

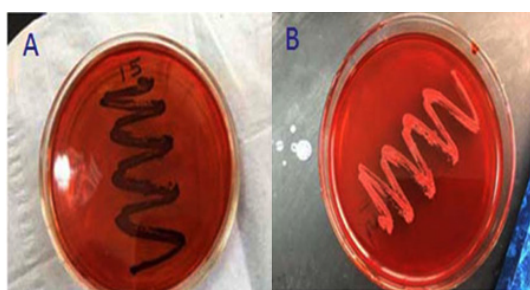


Fig. 3. A) CRA plate: Black colonies (positive). (B): Red smooth colonies (negative)

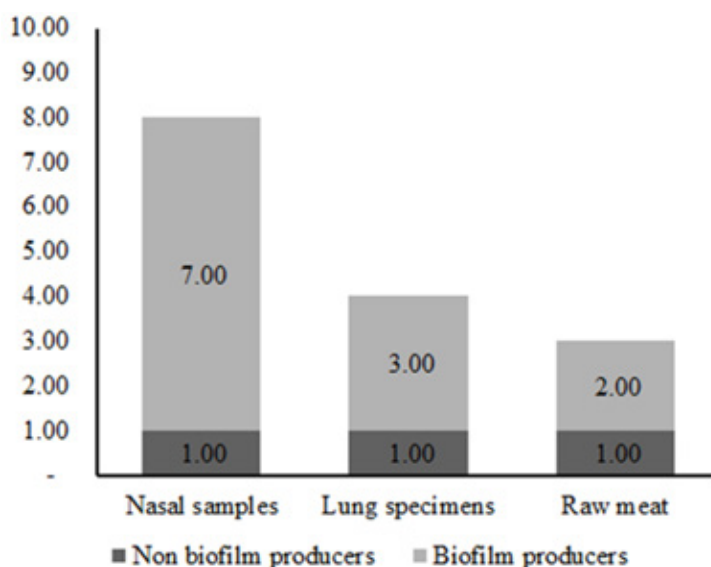


Fig. 4. MTP test of 15 MRSA isolates. Positive samples (OD >0.6) were 87.5%, 75% and 5% in nasal, lung and raw meat respectively

respectively (Figure 2). From the 130 samples, 75 samples were *S. aureus*, 15 were positive for both *nuc* and *mec* Agenes and were proved as MRSA isolates. Strains for *S. aureus* MRSA were identified by the expression of the *mecA* gene which confirmed the disc diffusion test results. Moreover, *nuc* gene was detected in all MRSA isolates (15/15).

CRA test was used for biofilm detection, black colonies with slimy texture revealed positive biofilm MRSA producers, while red colonies indicated none biofilm producing strains (Figure 3A-B). All *S. aureus* MRSA were cultured on CRA to differentiate between biofilm and none biofilm producers. The obtained results denoted that 12 out of 15 examined MRSA isolates (80%) were considered biofilm producers (black color) meanwhile three isolates (20%) were red color that means negative biofilm producers (Figures 3 and 4). MTP test confirmed the results obtained by CRA. The obtained data was the same as that of CRA as seen in Figure 5.

S. aureus MRSA biofilms were exposed to garlic extract (gWE). The results of gWE reported that *S. aureus* MRSA biofilm was inhibited significantly after initial incubation with 25 mg/ml garlic extract (Fig. 5). In parallel, Figure 6 showed the inhibitory effect of *Lactobacilli biosurfactants* on biofilm generation by MRSA. Compared to control, *S. aureus* MRSA biofilm was repressed by

Lactobacilli biosurfactants at 50 and 100 mg/ml for 18 hrs.

DISCUSSION

MRSA has gained increasing importance in veterinary medicine in the past 2 decades, as MRSA shows resistance not only β -lactams but also other classes of antimicrobials^{24,25}. The distribution of MRSA and its several antibiotics resistance are growing worldwide²⁶. The prevalence of MRSA in large ruminant farms was 34% in milk samples collected from Pakistan²⁷. The presence of MRSA in samples of meat was previously confirmed²⁸⁻³⁰, while it varies greatly by geographical location. Lower results from retail meat (< 5%) were reported before^{31,32}, while Similar results (10-12 %) were shown in other studies^{33,34}. Other, confirmed that MRSA prevalence was 31.8% in cattle, 29.8% of sheep, and 11.5% in goats in Jordan³⁵.

Our findings showed that MRSA isolates were detected in 20% of our samples and the difference from other results may be attributed to the origin of samples used as they used milk samples while ours are nasal, lung and raw meat samples. The *mecA* gene detection by triplex PCR technology is a gold tool to detect MRSA strains compared to conventional disc diffusion test³⁶.

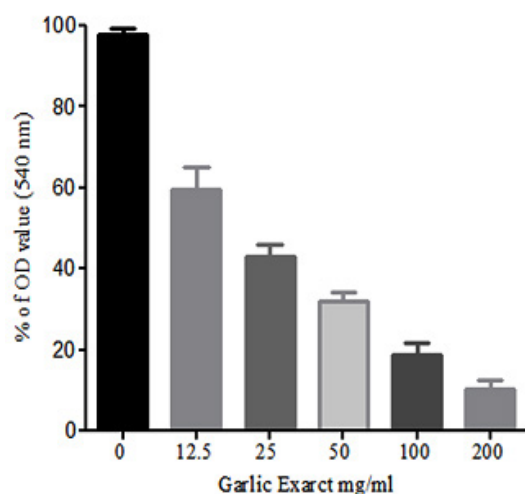


Fig. 5. The anti-biofilm activity of garlic water extract (gWE). The inhibitory effect of gWE was dose dependent manner

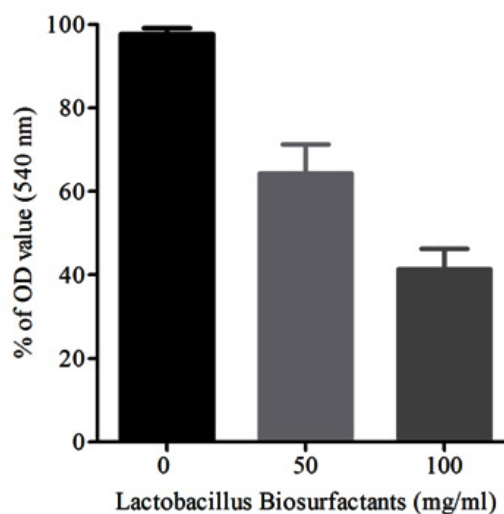


Fig. 6. Anti-biofilm activity of *Lactobacillus Biosurfactants*. The inhibitory effect of *Lactobacillus Biosurfactants* was dose dependent manner

During the past two decades, scientists confirmed the increase in prevalence of *mecA* gene with multiple antibiotics resistance³⁷.

Nearly, all *mecA* positive isolated *S. aureus* strains exhibited resistance to oxacillin and cefoxitin³⁸. These results go ahead with our reported results, where, all isolated MRSA with *mecA* gene were all resistant to oxacillin and cefoxitin using disc diffusion test. The *nuc* gene was found also in all isolated MRSA strains, as it targeted *S. aureus* specific isolates and was more species specific than 16S rRNA (*S. species specific*)³⁹. The high percent reported may be attributed to the high use of antibiotics as growth promoters in animals. Same was confirmed in a previous reported study in Jordan (29.8%)³⁵.

The biofilms helps the bacterium to resist death and still survive within the host, then cause chronic or persistent infections⁴⁰. Biofilm formation plays a vital role in pathogenicity, virulence and antimicrobial resistance to antibiotics⁴¹. The ability of pathogenic bacteria, such as *S. aureus*, to produce biofilm is the cause for persistent infections⁴². Bacteria producing biofilm are resistant to antibiotics, compared with non-biofilm producers⁴³. The degree of biofilm formation by MRSA depends on the intercellular adhesion molecules production, named intercellular adhesion (*ica*) loci that include *icaA*, *icaB*, *icaC*, and *icaD* genes⁴⁴. The process of biofilm formation consists of two independent processes: bacteria initially, attached to a solid surface. followed by proliferation and accumulation of bacteria cells, and thus resulted in biofilm formation and maturation⁴⁵.

There are different methods for phenotypic detection of slime production as CRA which used in our study and considered rapid reliable qualitative test. CRA contains congo red dye that can detect the polysaccharides on the cell wall of both Gram positive and Gram negative bacteria¹⁶. The obtained results showed that (12/15) of MRSA isolates were considered as biofilm producers (black color) (80%). CRA was used in Previous studies and the biofilm producing MRSA (black color) was confirmed and was with a percentage of 85.1%⁴⁶. MTP is another phenotypic test but more convenient and quantitative than CRA depending on measuring of OD using spectrophotometer and can detect *S. aureus* cell wall polysaccharides

adhering to the polystyrene plate directly. The obtained results are coincided with that of CRA. Previous studies showed that (93/94) MRSA isolates were biofilm producers using the microtitre plate test⁴⁷.

The anti-biofilm impact of water extract garlic (gWE) was investigated. A reduction in biofilm generation was reported at a concentration equivalent to 0.25 mg/ml gWE. The effect of gWE can be attributed to its active components that stifled the biofilm formation⁴⁸. In parallel, previous reports confirmed that *bio-surfactants* separated from *Lactobacilli* strains has anti-biofilm activity against MRSA. This findings that potentiate our results increase the usage of these natural products as an alternate promising therapies for treatment of *S. aureus* MRSA strains⁴⁹.

CONCLUSIONS

Current study characterized and determined the prevalence of *S. aureus* MRSA in Turabah, Saudi Arabia using well established microbiological and molecular techniques. Moreover, confirmed the anti-biofilm activity of garlic and *lactobacillus biosurfactants* as promising medications for treatment of *S. aureus* MRSA in animals.

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