Ascorbic acid augments colony spreading by reducing biofilm formation of methicillin-resistant Staphylococcus aureus

Zulfiqar Ali Mirani1*, Muhammad Naseem Khan 1, Anila Siddiqui 1, Fouzia Khan 2, Mubashir Aziz 3, Shagufta Naz 1, Ayaz Ahmed 4, Seema Ismat Khan 1

1 FMBRC, Microbiological Analytical Centre, Pakistan Council of Scientific and Industrial Research Laboratories Complex, Karachi, Pakistan
2 Department of Veterinary Pathology, Microbiology Section, BZU Multan, Pakistan
3 Dow University of Medical and Health Sciences, Karachi, Pakistan
4 PCMD, ICBBS, University of Karachi, Karachi, 75270, Pakistan

**Abstract**

**Objective(s):** Staphylococcus aureus is a Gram-positive pathogen, well known for its resistance and versatile lifestyle. Under unfavourable conditions, it adapts biofilm mode of growth. For staphylococcal biofilm formation, production of extracellular polymeric substances (EPS) is a pre-requisite, which is regulated by ica operon-encoded enzymes. This study was designed to know the impact of ascorbic acid on biofilm formation and colony spreading processes of S. aureus and MRSA.

**Materials and Methods:** The isolates of methicillin-resistant S. aureus (MRSA) used in present study were recovered from different food samples. Various selective and differential media were used for identification and confirmation of S. aureus. Agar dilution method was used for determination of oxacillin and ascorbic acid resistance level. MRSA isolates were re-confirmed by E-test and by amplification of mecA gene. Tube methods and Congo-Red agar were used to study biofilm formation processes. Gene expression studies were carried on real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

**Results:** The results revealed the presence of mecA gene belonging to SCCmec type IV along with agr type II in the isolates. In vitro studies showed the sub-inhibitory concentration of oxacillin induced biofilm production. However, addition of sub-inhibitory dose of ascorbic acid was found to inhibit EPS production, biofilm formation and augment colony spreading on soft agar plates. The inhibition of biofilm formation and augmentation of colony spreading observed with ascorbic acid alone or in combination with oxacillin. Moreover, gene expression studies showed that ascorbic acid increases agr expression and decreases icaA gene expression.

**Conclusion:** The present study concluded that ascorbic acid inhibits biofilm formation, promotes colony spreading and increases agr gene expression in MRSA.

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**Introduction**

*Staphylococcus aureus* is among the most prevalent causes of clinical infections worldwide and has gained substantial public attention due to increased mortality, associated with multi-drug resistance (1). Most of the *S. aureus* foodborne illnesses result from contamination of food by handlers, while low levels of the *S. aureus* may exist in many types of food items (2). Owing to its ability to produce a number of extracellular toxins such as capsular polysaccharides, staphylococcal enterotoxins (SEs), toxic shock syndrome toxin 1 (TSST-1) pantenol -valentine leukocidin (PVL) it is considered as one of the most important food-borne pathogen (3). Staphylococcal food poisoning, although generally self-limiting, is a major cause of illness worldwide (4). Moreover, the ability of *S. aureus* to express virulence factors that facilitate their adherence to surfaces followed by biofilm formation (5).

Biofilms are defined as adherent, matrix-enclosed bacterial populations resistant to antibiotics and physical treatments (6). The resistant nature of biofilm is a matter of great concern for global health care system and many studies have been conducted to explore anti-biofilm agents. Yet there are few studies on anti-biofilm properties of food additives, as well. Safe food additives having biofilm inhibitory activity would be desirable for use in controlling biofilms in the food environment. The food additives having reportedly antibacterial activity include ascorbic acid, a widely used additive that helps to prevent food spoilage. It mainly acts as an antioxidant, thereby preserve foods by inhibiting the effects of oxygen and hence can also be beneficial to health (7). Recently, a combination of ascorbic acid with lactic acid has been reported to inhibit the growth of *Escherichia coli* O157:H7 in food (8). Considering the potential of...
ascorbic acid as a food preservative and its antibacterial activity, the present study was designed to analyse the effect of ascorbic acid on foodborne isolates of MRSA.

**Materials and Methods**

**Identification of S. aureus**

In this study, total 20 biofilm producing isolates of MRSA were used. The subject MRSA isolates were recovered from different food commodities e.g. candies, spices mix, lentils, mayonnaise, meat and meat products. These isolates were identified by monitoring growth on differential and selective media e.g. Mannitol Salt Agar (Oxoid-Hampshire-England), Staph-Chromo Agar (Merck-Darmstadt, Germany), Staphylococcus 110 Agar (Oxoid-Hampshire-England), Baird Parker Agar (Oxoid-Hampshire-England), DNase Agar (Merck-Darmstadt, Germany) and Blood Agar (Oxoid-Hampshire-England). Staph Latex Kit (Pro-LAB, Wirral, UK) was used for confirmation.

**Determination of MIC for oxacillin**

Minimum inhibitory concentration (MIC) of oxacillin (Sigma-Aldrich,USA) was determined by Muller Hinton (MH) agar (Oxoid-Hampshire-England) dilution method (CLSI VET01-A4) (9) and was re-confirmed by E-test (AB-Biodisk-Soha, Sweden) according to the manufacturers instructions.

**Determination of MIC for ascorbic acid**

The antibacterial activity of ascorbic acid (Sigma-Aldrich, Steinheim, Germany) was determined by tube dilution method as described by Cursino et al. (10) with some modification e.g. brain heat infusion (BHI) was used instead of Muller Hinton (MH) agar. Ascorbic acid (2-128 µg/ml) was added to the BHI Broth (Oxoid-Hampshire-England) (pH 7.0). Tubes containing an identical amount of medium without ascorbic acid and tubes (separately) containing oxacillin and ascorbic acid were included in each assay. After 24-48 hr of incubation at 35 °C, the lowest concentration of ascorbic acid either separately, or in combination with oxacillin, that prevented the development of turbidity, was considered as the MIC.

**Phenotypic characterization of slime producing bacteria**

Biofilm formation was initially confirmed by Congo red agar method as described earlier (11). Briefly, BHI agar plates containing 50 g/l sucrose and 0.8 g/l Congo-red (Alfa Aesar-Karlsruhe, Germany) were supplemented with ascorbic acid (1 mg/ml) and sub-inhibitory concentration of oxacillin (pH 7.0) were prepared and streaked with subject strains and incubated aerobically for 24-48 hr at 35 °C. Plates containing medium only, and the plates separately containing the antibiotic or ascorbic acid were included as control. Positive results were indicated by black colonies with dry crystalline appearance. Weak slime producers usually remained pink, though occasional darkening at the center of colonies.

**Biofilm assay**

A qualitative assessment of biofilm formation by glass slides was evaluated by tube method. Two-inch piece of glass slide was submerged in tryptone soy broth (TSB) (Oxoid-Hampshire England) (pH 7.0) supplemented with sub-inhibitory doses of oxacillin and ascorbic acid, inoculated with 0.1 ml of fresh culture of the subject isolate and incubated at 35 °C for 24 – 72 hr. Tubes containing medium only, and tubes separately containing the antibiotic or ascorbic acid were included as control. After incubation, glass slides were taken out from broth and washed with phosphate buffer saline (pH 7.0) to remove unbound cells and debris; biofilms were fixed with acetic acid for 15 min, stained with 3% crystal violet and observed under the microscope (14). Each experiment was repeated four times to check the accuracy and precision of results.

**Effect of ascorbic acid and oxacillin on pre-formed biofilms**

After induction at 35 °C and maturation of biofilms (usually after 48 hr), the glass slides were removed and washed three times with PBS (pH 7.0) to remove unbound cells and debris. The glass slides were incubated in TSB supplemented with ascorbic acid (at MIC level of subject isolate) oxacillin (at MIC level of subject isolate), ascorbic acid + oxacillin and saline suspension for 6 hr at 35 °C. The succession of the biofilm was measured by monitoring OD every hour for 6 hr. The number of viable cells disperse from biofilm consortia was determined as described previously (12).

**Quantification of biofilms**

Biofilm formation was quantified by the addition of 2×200 µl of 95% ethanol as described by O‘Toole et al (13) and A563 was recorded with spectrophotometer (Nicollet Evolution 300 BB).

**Colony spreading**

Colony spreading properties of subject isolates were studied as described by Kaito & Sekimizu (15).

**Polymerase chain reaction (PCR)**

For molecular studies, genomic DNA and RNA were isolated using the DNase Kit (Qiagen, Hilden-Germany), following the manufacturer’s instruction. PCR amplification of icaA, mecA and agr genes was performed with an MWG Thermal Cycler in a volume of 50 µl of Promega (Madison, WI 53711 USA) Master mix. Primers and conditions for the PCR and gene expression have been described in our previous works (14, 18). 16S rDNA was used as internal control for gene expression and species identification.
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Figure 1. Colony spreading and biofilm formation on congo-red agar. (A) Biofilm formation and EPS production in the presence of oxacillin on congo-red agar plate. (B) Control i.e. congo-red and tryptone soy agar plate without any supplement. (C) Colony spreading without EPS production on congo-red agar plate supplemented with sub-inhibitory dose of ascorbic acid. (D) Colony spreading without EPS production on congo-red agar plate supplemented with sub-inhibitory doses of ascorbic acid and oxacillin

**Electron microscopy**

Scanning electron microscopy was carried out to analyse the production of extra-cellular matrix material after exposure to oxacillin. Biofilm slides were divided into 4 mm sections and washed with distilled water to remove debris negatively stained with 0.2% uranyl acetate for 30 sec. These 4 mm slide sections showed the presence of biofilm material when examined directly in a GOEL-JSM-6380A Electron Microscope (14).

**Results**

In the present study, 20 isolates of *S. aureus* were studied that were recovered from different food commodities. These isolates were identified as *S. aureus* on the basis of colonial characters i.e. black colonies with opaque zone on Baird-Parker agar with egg yolk tellurite, Mannitol fermentation on mannitol salt agar. Further confirmation was made by positive reaction on Prolex Latex Agglutination System and amplification of 16S rRNA.

**MIC of oxacillin and ascorbic acid**

Oxacillin susceptibility testing results revealed that these isolates were MRSA. Out of 20 isolates, six strains exhibited high-level oxacillin resistance (MIC 64 µg/ml), seven isolates exhibited intermediate oxacillin resistance (MIC 32 µg/ml) while remaining seven isolates showed low-level oxacillin resistance (MIC 8-16 µg/ml) (Table 1). Majority of the isolates (n12 out of 20 isolates) exhibited low-level resistance to ascorbic acid (MIC 4-8 µg/ml) (Table 1). In seven (7) isolates ascorbic acid MIC was 16µg/ml and only one isolates inhibited at 32µg/ml (Table 1). Moreover, PCR studies showed that these isolates of MRSA carry mecA gene, belong to SCCmecA type IV and harbor agr type II (Table 1).

**Biofilm formation**

Phenotypic study revealed that sub-inhibitory doses of oxacillin induced biofilms in the isolates. This was confirmed by typical characters of biofilm forming colonies with rough, dry and crystalline appearance on congo-red agar and scanning electron microscopy (Figure 2). Electron micrographs showed adhesive cells surrounded with extra-cellular matrix material. This process was started in an organized way; initially (16th to 18th hr of incubation) a monolayer covered the surface of glass slide and cells were interconnected by the help of extra-cellular matrix material. The maximum optical density (OD) of biofilm was achieved after 48 hr of incubation at 35 °C and a multi-layer consortium was observed (Figure 2). It was also noteworthy that the addition of ascorbic acid inhibited the EPS production as indicated by congo-red agar plate assay (Figure 1) and biofilm formation. A detailed study of the subject isolates showed that biofilms tolerate higher doses of ascorbic acid and oxacillin as compared to planktonic population (Table 1). However, high dose of ascorbic acid has capacity to disperse mature biofilms more effectively and quickly as compared to oxacillin. Ascorbic acid alone or in combination with oxacillin, dispersed mature biofilms in 90 min, whereas, it required 4-6 hr to achieve similar results with oxacillin alone. Moreover, after 90 min...
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Table 1. Biofilm formation and impact of oxacillin (Oxa) and ascorbic acid (AA) on biofilm formation properties of subject isolates of MRSA SCCmecA and agr typing of MRSA

| Isolate No | SCC mecA type | Agr type | Biofilm OD at sub-MIC of oxacillin | Biofilm OD after treatment at 48 hr | Recovery of viable population (CFU) after biofilm treatment at 48 hr | Oxacillin MIC in µg/ml | Ascorbic acid MIC in µg/ml |
|------------|----------------|---------|-----------------------------------|-----------------------------------|---------------------------------------------------------------|------------------------|---------------------------|
| 1. IV II   | 0.09 0.96      | 0.7 0.13 0.25 | 110±5 17 19 54 | 64 128 04 16 |
| 2. IV II   | 0.08 0.92      | 0.11 0.13 0.26 | 085±5 22 15 47 | 64 128 04 16 |
| 3. IV II   | 0.23 0.89      | 0.19 0.16 0.34 | 101±5 33 25 44 | 64 128 08 32 |
| 4. IV II   | 0.27 0.73      | 0.32 0.34 0.42 | 129±5 12 16 42 | 64 128 08 32 |
| 5. IV II   | 0.17 0.86      | 0.25 0.22 0.33 | 077±5 19 16 47 | 32 128 16 32 |
| 6. IV II   | 0.21 0.80      | 0.26 0.24 0.44 | 103±5 27 33 63 | 32 128 16 32 |
| 7. IV II   | 0.22 0.79      | 0.11 0.14 0.32 | 156±5 09 12 78 | 32 128 16 32 |
| 8. IV II   | 0.35 0.76      | 0.54 0.49 0.55 | 177±5 25 25 89 | 32 128 16 32 |
| 9. IV II   | 0.33 0.75      | 0.51 0.53 0.61 | 180±5 14 26 66 | 32 128 16 32 |
| 10. IV II  | 0.55 0.87      | 0.45 0.47 0.45 | 099±5 12 07 57 | 16 128 16 32 |
| 11. IV II  | 0.54 0.81      | 0.36 0.33 0.39 | 087±5 08 13 63 | 16 64 08 16 |
| 12. IV II  | 0.55 0.86      | 0.31 0.30 0.44 | 097±5 15 19 45 | 08 64 04 32 |
| 13. IV II  | 0.25 0.81      | 0.33 0.31 0.43 | 093±5 19 11 43 | 08 64 04 32 |
| 14. IV II  | 0.09 0.85      | 0.22 0.26 0.33 | 098±5 13 17 43 | 64 192 32 64 |
| 15. IV II  | 0.08 0.97      | 0.31 0.34 0.51 | 107±5 18 14 69 | 64 256 16 32 |
| 16. IV II  | 0.23 0.82      | 0.19 0.16 0.49 | 136±5 26 32 77 | 32 256 08 16 |
| 17. IV II  | 0.27 0.79      | 0.22 0.22 0.39 | 132±5 18 15 83 | 32 256 08 16 |
| 18. IV II  | 0.17 0.81      | 0.33 0.35 0.22 | 119±5 25 19 54 | 16 128 08 16 |
| 19. IV II  | 0.21 0.83      | 0.16 0.18 0.36 | 127±5 27 23 67 | 08 64 04 32 |
| 20. IV II  | 0.22 0.76      | 0.34 0.39 0.44 | 098±5 33 21 52 | 08 128 04 32 |

**Effect of ascorbic acid on agr and icaA gene expression**

Gene expression studies of subject isolates of the *S. aureus* showed that sub-inhibitory doses of ascorbic acid augment *agr* gene expression and reduce *icaA* gene expression. Highest *agr* gene expression was noticed in the presence of sub-inhibitory dose of ascorbic acid (Figure 3). Contrary to this, sub-inhibitory doses of oxacillin augment *icaA* gene expression (Figure 4). When sub-inhibitory doses of oxacillin and ascorbic acid were used in combination, a minor reduction was noticed in gene expression of *agr* whereas a remarkable decrease in *icaA* gene expression was noticed. This indicated induction of *agr* by ascorbic acid.

![Figure 3: agr gene expression of MRSA after exposure to sub-inhibitory doses of ascorbic acid (AA), Oxacillin (Oxa) and oxacillin+ascorbic acid (AA+Oxa)](image)

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Figure 4. icaA gene expression in MRSA isolates after exposure to sub-inhibitory doses of ascorbic acid (AA), Oxacillin (Oxa) and oxacillin+ascorbic acid (AA+Oxa)

Discussion

MRSA is one of the leading causes of foodborne illness (16). This is one of the most versatile bacteria, having the tendency to change its lifestyle according to the environment e.g. planktonic to biofilm (17). In our previous work it was reported that oxacillin induces biofilm mode of life in MRSA, which are highly resistant to conventional treatment options (14). In search of controlling biofilm, during present study different compounds have been tried i.e. fatty acids, ascorbic acid and variety of plant extracts. Ascorbic acid was found to inhibit biofilm formation and also dispersed pre-established biofilm of MRSA. After exposure to oxacillin a coordinated interaction was noticed in resistant population. At initial stage of biofilm formation (16th to 18th hr) cells showed an interconnected and organised monolayer on glass surface. At this stage the icaA gene expression was also on its peak level. In our previous work (14), it was confirmed that oxacillin has a regulatory effect on icaA gene expression and induces the icaA dependent polysaccharide intracellular adhesion (PIA) production and biofilm formation (14). Ascorbic acid inhibits icaA dependent PIA production pre-requisite for cell to cell interaction in biofilm consortia. This was confirmed by the reduction in icaA gene expression of subject isolates after exposure to ascorbic acid. It was also noticed that ascorbic acid removes EPS and disperse pre-formed biofilms. After 90 min treatment of biofilm consortia, majority of the cells were unable to survive. However, a slow growing and non-pigmented minor population of biofilm consortia tolerated the ascorbic acid treatment. In our previous work (14), it was mentioned that mature biofilms consortium of subject isolates of MRSA harbor heterogeneous population i.e. wild type and small colony variants (SCVs). The recovery of these phenotypes was higher (6-8 times) when mature biofilm treated with oxacillin. These highly resistant phenotypes were unable to re-adopt biofilm mode of growth, however sub-inhibitory dose of ascorbic acid augment colony spreading. This is termed as passive type of movement across agar surface. This process is regulated by agr system in S. aureus. Tsompanidou et al (19) described that agr system is required for the movement of S. aureus over surface-air interfaces by colony spreading. Gene expression studies showed that sub-inhibitory doses of ascorbic acid augment agr gene expression by down-regulating icaA expression. In our previous study (14) the role and relation of agr and ica operon in biofilm formation and dispersion has been defined. It was mentioned that oxacillin induces ica dependent biofilm formation by reducing agr expression which is involved in biofilm dispersion (18). This phenomenon has been well defined by Tsompanidou et al (19) and Horswill (20), where they have mentioned that agr-deficient Staphylococci produce strong biofilms and agr+ isolates relatively have high potential for colony spreading over surfaces.

Conclusion

In this study it has been noticed that oxacillin induces icaA dependent biofilm mode of life in MRSA. However, ascorbic acid reduces biofilm formation by inhibition of icaA gene expression and augments colony spreading in subject isolates.

Acknowledgment

We are thankful to Mr M Yousf Khan of University of Karachi for providing SCM and RT-PCR facilities for this study.

Conflict of interest

All authors confirm that there are no conflicts of interest.

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