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

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ARTICLE INFO	ABSTRACT
Article type: Original article *Article history:* Received: Dec 13, 2016 Accepted: Sep 28, 2017 *Keywords:* Ascorbic acid Biofilms Colony spreading Methicillin-resistant *S. aureus* *Staphylococcus aureus*	***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 SCC*mecA* 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.

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) panton – 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

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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-Solna, 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 A_{563} 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.
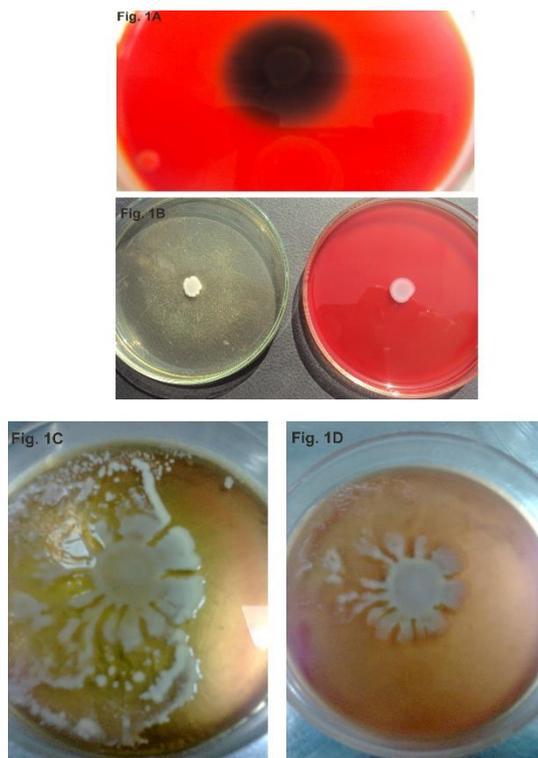
Figure 1. Colony spreading and biofilm formation on congo-red agar. (A) Biofilm formation and EPS production in the presence of oxacillin on congo-red agar plate. (B) Control i.e. congo-red and tryptone soya agar plate without any supplement. (C) Colony spreading without EPS production on congo-red agar 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

Figure 2. Scanning electron micrographs of biofilm consortia (A) after exposure to sub-inhibitory concentration of oxacillin isolate (n8) produced EPS and adapted biofilm mode of growth. (B) Cells devoid of EPS in the presence of 1 mg/ml ascorbic acid. (C) Multicellular aggregates of biofilms (isolate n8) embedded in EPS after exposure to sub-inhibitory concentration of oxacillin (D) Control growth on TSA plate

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
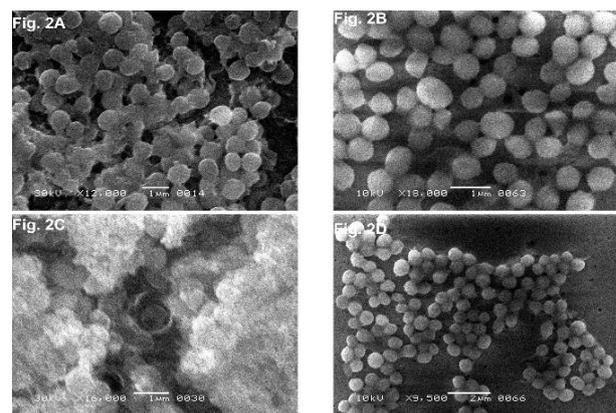
64 μg/ml), seven isolates exhibited intermediate oxacillin resistance (MIC 32 μg/ml) while remaining seven isolates showed low-level oxacillin resistance (MIC 8 -16 μg/ml) (Table 1). Majority of the isolates (n12 out of 20 isolates) exhibited low-level resistance to ascorbic acid (MIC 4 to 8 μg/ml) (Table 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

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	
			24 H	48 H	AA	AA+ Oxa	Oxa	Control	AA	AA+ Oxa	Oxa	24 H	48 H	24 H	48 H
1.	IV	II	0.09	0.96	0.07	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	08
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

treatment with ascorbic acid, 99% of mature biofilms disperse and cells were unable to survive. Although, both of these agents could killed majority of the cells in biofilms, nonetheless, a layer of highly resistant and strongly adhered cells on glass surface survived. Furthermore, the results for viable count showed that ascorbic acid suppressed EPS production and biofilm formation. However, some highly resistant phenotypes survived and tolerated the toxic effect of ascorbic acid (Table 1).

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.
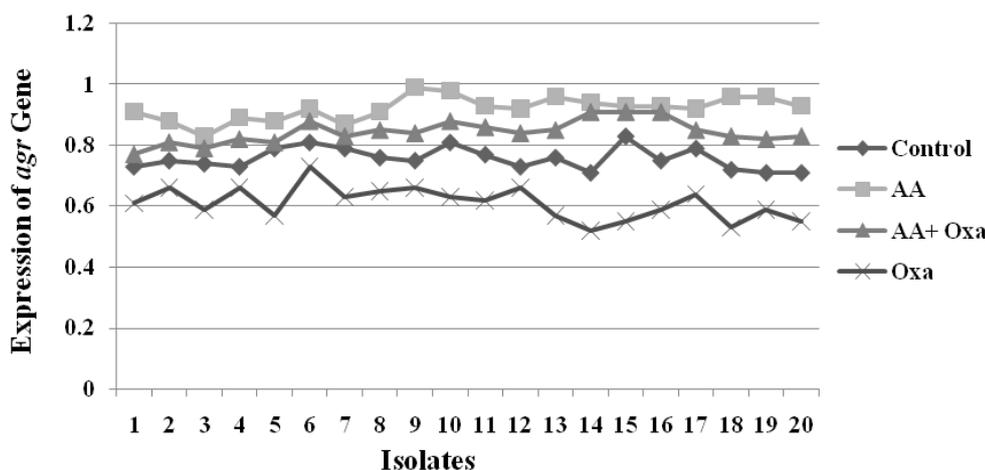

Figure 3. agar gene expression of MRSA after expoure to sub-inhibitory doses of ascorbic acid (AA), Oxacillin (Oxa) and oxacillin+ascorbic acid (AA+Oxa)
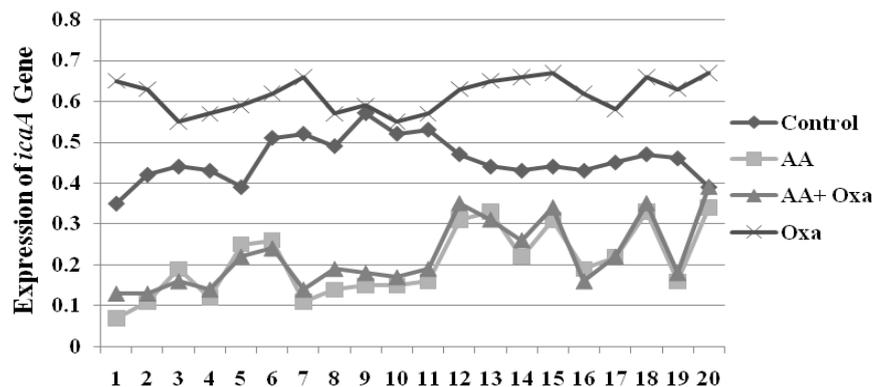
Figure 4. *icaA* gene expression in MRSA isolates after expoure to sub-inhibitory doses of ascorbic acid (AA), Oxacillin (Oxa) and oxacillin+ascorbic acid (AA+Oxa)

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 is 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* operon 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 *ica* dependent biofilm mode of life in MRSA. However, ascorbic acid reduces biofilm formation by inhibition of *ica* gene expression and augments colony spreading in subject isolates.

Acknowledgment

Conflict of interest

All authors confirm that there are no conflicts of interest.

References

1. Waters AE, Contente-Cuomo T, Buchhagen J, Liu CM, Watson L, Pearce K, Foster JT, Bowers J, Driebel EM, Engelthaler DM, Keim PS, Price LB. Multidrug-resistant

Staphylococcus aureus in US meat and poultry. Clin Infect Dis 2011; 52: 1227–1230.

2. Le Loir Y, Baron F and Gautier M. *Staphylococcus aureus* and food poisoning. Genet Mol Res 2003; 2: 63–76.

3. Gordon RJ, Lowy FD. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. Clin Infect Dis 2008; 46: (Suppl 5) S350-359.

4. Chin J. Control of communicable diseases manual. 17th ed. Baltimore: United Book Press, Inc. 2000.

5. Hudson MC, Ramp WK, Frankenburg KP. *Staphylococcus aureus* adhesion to bone matrix and bone-associated biomaterials. FEMS Microbiol Lett 1999; 173: 279–284.

6. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 2002; 15: 167-193.

7. Russell AD. Mechanisms of bacterial resistance to non-antibiotics: food additives and food and pharmaceutical preservatives. J Appl Bacteriol 1990; *71*: 191–201.

8. Tajkarimi M and Ibrahim SA. Antimicrobial activity of ascorbic acid alone or in combination with lactic acid on *Escherichia coli* O157: H7 in laboratory medium and carrot juice. Food Control 2011; 22: 801-804.

9. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals—Fourth Edition: Approved Standard VET01-A4. CLSI, Wayne, PA, USA, 2013.

10. Cursino L, Chartones ES, Nascimento AM. Synergic interaction between ascorbic acid and antibiotics against *Pseudomonas areuginosa*. Braz Arch Biol Technol 2005; 4: 31-38.

11. Arciola CR, Baldassarri L, Montanaro L. Presence of *icaA* and *icaD* Genes and slime production in a collection of

Staphylococcal strains from catheter associated infections. J Clin Microbiol 2001; 39: 2151-2156.

12. Zhao Y, Caspers MP, Metselaar KI, de Boer P, Roeselers G, Moezelaar R, *et al.* Abiotic and microbiotic factors controlling biofilm formation by thermophilic spore formers. Appl Environ Microbiol 2013; 79(18):5652–60.

13. O'Toole GA, Kolter R. The initiation of biofilm formation in *Pseudomonas fluorescence* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 1998; 28: 449-461.

14. Mirani ZA, Aziz M, Khan SI. Small colony variants have a major role in stability and persistence of *Staphylococcus aureus* biofilms. J Antibiot 2015; 68:98-105.

15. Kaito C, Sekimizu K. Colony spreading in *Staphylococcus aureus.* J Bacteriol 2007; 189: 2553–2557.

16. Ortega E, Abriouel H, Lucas R & Galvez A. Multiple roles of *Staphylococcus aureus* enterotoxins: pathogenicity, superantigenic activity, and correlation to antibiotic resistance. Toxins 2010; 2: 2117-2131.

17. Otto M. Staphylococcal biofilms. Curr Top Microbiol Immunol. 2008; 322: 207–228.

18. Mirani, Z.A., Aziz, M., Khan, M.N., Lal, I., Hassan, N.U., and Khan, S.I. Biofilm formation and dispersal of *Staphylococcus aureus* under the influence of oxacillin. Microb Pathog 2013; 61–62C: 66–72.

19. Tsompanidou E, Sibbald MJ, Chlebowicz MA, Dreisbach A, Back JW, *et al.* Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*. J Bacteriol 2011; 193: 1267–1272.

20. Boles BR, Horswill AR. Staphylococcal biofilm disassembly. Trends Microbiol 2011; 19: 449–455.