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ORIGINAL RESEARCH

Synergistic Anti-Biofilm and Bactericidal Activity of Ethanol and Chlorhexidine Combined with EDTA Against *Staphylococcus aureus* Isolates from Healthcare-Associated Carriers

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Introduction: Healthcare-associated infections (HAIs), particularly in dialysis units, remain a significant challenge due to the frequent use of invasive devices and the immunocompromised status of patients. Among the most concerning pathogens is *Staphylococcus aureus*, known for its multidrug resistance and biofilm-forming capacity. This study aimed to investigate the antibiofilm effects of ethanol, chlorhexidine, and EDTA, individually and in combination, against *S. aureus* isolates from hospital carriers in the dialysis department of Qazvin teaching hospitals, Qazvin, Iran.

Methods: A total of 400 samples were collected from nasal cavities, fingernails, patient beds, and dialysis machines. Isolates were identified by biochemical tests and PCR. Antibiotic susceptibility was evaluated via disk diffusion and E-test, with mupirocin resistance determined by mupA and mupB detection. Biofilm formation was assessed using the microtiter plate assay with crystal violet staining. The antimicrobial and antibiofilm activities of a combined solution of chlorhexidine, ethanol, and EDTA were analyzed using broth microdilution and scanning electron microscopy (SEM).

Results: 70 *S. aureus* isolates were analyzed for antibiotic resistance and biofilm formation. High resistance rates were observed to ciprofloxacin (62.8%), doxycycline (57.1%), and tetracycline (54.2%), while mupirocin resistance was detected in 4.2% of isolates (*mupA* positive). Multidrug resistance (MDR) was found in 68.6% of isolates. Strong biofilm formation was observed in 90% of isolates and was significantly associated with MDR. The combination of chlorhexidine and EDTA exhibited potent antibiofilm activity (FICI $\leq$ 0.5), with SEM imaging confirming disruption of biofilm structure and bacterial cell integrity.

Conclusion: Our findings indicate a high prevalence of multidrug-resistant and strong biofilm-producing *S. aureus* in dialysis units. Among the tested disinfectant combinations, chlorhexidine in combination with EDTA demonstrated substantial antibiofilm and antimicrobial effects, whereas EDTA and ethanol alone showed no significant activity. These results highlight the potential of synergistic disinfectant combinations to disrupt biofilm structures and suggest the need for further studies to evaluate their applicability in clinical settings.

Keywords: *Staphylococcus aureus*, biofilm formation, dialysis units, ethanol, chlorhexidine

Introduction

Healthcare-associated infections (HAIs) continue to challenge modern medicine, particularly in specialized clinical environments such as dialysis units.[1] These settings are characterized by frequent invasive procedures, prolonged patient exposure to medical devices, and a high prevalence of immunocompromised individuals.[2] Among the pathogens that dominate this landscape, *S. aureus* has garnered particular attention due to its dual role as a commensal organism and an

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opportunistic pathogen.[3] Its ability to colonize human skin and mucosal surfaces, coupled with its arsenal of virulence factors and resistance mechanisms, renders it a formidable threat in hospital environments.[4] *S. aureus* is notorious for its capacity to transition from benign colonizer to aggressive invader, a process mediated by a complex interplay of genetic and environmental factors that regulate the expression of surface proteins, toxins, and enzymes.[5]

One of the most clinically significant features of *S. aureus* is its ability to form biofilms, structured microbial communities embedded in a self-produced extracellular matrix.[6] Biofilms are not merely passive aggregations of cells; they represent a highly organized and dynamic system that confers protection against host immune responses and antimicrobial agents.[7] In the context of dialysis wards, biofilms can form on catheters, dialysis machines, and other medical surfaces, serving as reservoirs for persistent infections and facilitating nosocomial transmission.[8] The widespread use of antibiotics in clinical settings has exerted selective pressure, leading to the emergence of multidrug-resistant (MDR) strains.[9] The increasing prevalence of MDR *S. aureus* strains, reported in several clinical settings, poses an additional threat by limiting treatment options and enhancing persistence through strong biofilm formation. Resistance to commonly used antibiotics such as ciprofloxacin, tetracycline, and linezolid has been increasingly reported, complicating treatment regimens and elevating the risk of therapeutic failure.[10,11]

In light of these challenges, the role of chemical disinfectants in infection control has become increasingly important. Chlorhexidine and ethanol are widely used for surface and skin disinfection due to their broad-spectrum antimicrobial activity.[12] However, their efficacy against biofilm-embedded bacteria is often limited, as biofilms act as physical and chemical barriers, impeding the penetration of disinfectants and reducing their bactericidal potential.[13] This has prompted the exploration of adjunctive agents such as EDTA, a chelating compound capable of destabilizing biofilm matrices by sequestering divalent cations.[14] EDTA disrupts the structural integrity of the biofilm, enhancing the permeability and efficacy of co-administered disinfectants.[15] Notably, chlorhexidine is one of the most common compounds for disinfecting hospital environments; by incorporating EDTA, lower concentrations of chlorhexidine can be used while achieving greater efficacy, offering a cost-effective strategy for infection control. The synergistic interaction between EDTA and conventional disinfectants represents a promising avenue for biofilm eradication.[16] EDTA may potentiate antiseptic activity through chelation of divalent cations (Ca^{2+} and Mg^{2+}), leading to destabilization of the biofilm extracellular polymeric matrix, increased membrane permeability, and enhanced bacterial susceptibility to antimicrobial agents.

By targeting the biofilm's extracellular matrix and weakening its defenses, these combinations may overcome the limitations of monotherapy and achieve more effective microbial control.[17] This approach is particularly relevant in dialysis wards, where biofilm-associated contamination poses a persistent threat to patient safety.[18]

This study aims to assess the effects of different concentrations of ethanol, chlorhexidine, and EDTA, individually and in combination, on biofilm formation and antibiotic resistance in *S. aureus* strains isolated from dialysis patients and healthcare carriers in the wards of Qazvin hospitals.

Materials and Methods

Sampling

Between November 2023 and September 2024, a total of 400 samples were collected from hospitalized hemodialysis patients and healthcare personnel in teaching hospitals of Qazvin, Iran. Specifically, 208 samples were obtained from 104 hemodialysis patients (nail and nasal swabs), 146 samples from 73 healthcare personnel (nail and nasal swabs), and 46 samples from hospital surfaces, patient beds and linens, and dialysis devices.

Nasal swab samples were collected from both dialysis patients and healthcare personnel working in the different wards where dialysis patients were admitted. Sampling was performed using sterile swabs moistened with saline, targeting the anterior nares of both nostrils. Other sampling sites included the fingernails, patient beds, and dialysis machines. Swabs were immediately placed into Brain Heart Infusion (BHI) broth containing 6.5% NaCl (Condalab, Spain) and transported to the microbiology laboratory for culture. Isolates were identified as *S. aureus* based on Gram staining, catalase test, DNase activity, coagulase test (both slide and tube methods), and growth on Mannitol Salt Agar (MSA; Condalab, Spain).[19]

Molecular Confirmation of S. aureus

Following phenotypic identification, *S. aureus* isolates were genotypically confirmed. Genomic DNA extraction was performed using an ultrasonic disruption method (sonication). This technique involves lysing bacterial cells with high-frequency sound waves, followed by heating to ensure complete lysis and separation of cellular debris. Species-level identification was achieved using polymerase chain reaction (PCR) with *S. aureus*-specific primers targeting the nuclease (*nuc)* gene. The primers used are listed in Table 1. PCR was performed in a total volume of 25 µL containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 1 U Taq DNA polymerase (Thermo Fisher, USA), and 2 µL of template DNA. The thermal cycling program included an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were subsequently visualized using 1% agarose gel electrophoresis to confirm the presence of the target band, providing reliable differentiation from other coagulase-positive staphylococci.[19]

Antibiotic Susceptibility Testing (AST)

AST of the *S. aureus* isolates was performed using both the disk diffusion method and the E-test, in accordance with the CLSI guidelines (2025). For the disk diffusion assay, antibiotic disks were placed on the inoculated plates, including cefoxitin (FOX, 30 µg), ciprofloxacin (CP, 5 µg), doxycycline (D, 30 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), chloramphenicol (C, 30 µg), linezolid (LZ, 30 µg), erythromycin (E, 15 µg), clindamycin (CC, 2 µg), rifampin (RA, 30 µg), cefazolin (CZ, 30 µg), and tetracycline (TE, 30 µg) (Padtanteb, Iran).

Methicillin-resistant *S. aureus* (MRSA) strains were phenotypically identified by examining their susceptibility to cefoxitin.[22] Plates were incubated at 35 ± 2°C for 18–20 h, and inhibition zones were interpreted as susceptible, intermediate, or resistant according to CLSI criteria. Furthermore, minimum inhibitory concentrations (MICs) of Mupirocin were determined using E-test strips (Mast Group Ltd., UK) for all *S. aureus* isolates according to the CLSI 2025 guideline.[23] Multidrug resistance (MDR) was defined as resistance to at least one agent in three or more antimicrobial classes.

Molecular Detection of Drug and Detergent Resistance Genes

For genotypic confirmation of MRSA strains, PCR was performed to detect the presence of *mecA* and *mecC* genes, which are associated with methicillin resistance. Additionally, to confirm resistance to mupirocin, PCR was used to identify the *mupA* and *mupB* genes. Also, PCR was carried out to detect the presence of disinfectant resistance genes *(qacA/B and smr*) among the *S. aureus* isolates using specific primers previously reported.[21] PCR was performed in a total volume of

Table 1 The Primers Used in the Study

Primer Sequence (5′ → 3′)		Target Gene	Size (bp)	Annealing	Reference
Confirmatory	F: 5'- GCGATTGATGGTGATACGGTT −3' R: 5'- AGCCAAGCCTTGACGAACTAAAGC −3'	*nuc*	279	60	[19]
Drug Resistance	F: 5'- TATATTATGCGATGGAAGGTTGG −3' R: 5'- AATAAAATCAGCTGGAAAGTGTTG −3'	*mupA*	456	56	[20]
	F: 5'- CTAGAAGTCGATTTTGGAGTAG −3' R: 5'- AGTGTCTAAAATGATAAGACGATC −3'	*mupB*	674	56	[20]
	F: 5'- AAAATCGATGGTAAAGGTTGGC −3' R: 5'- AGTTCTGCAGTACCGGATTTGC −3'	*mecA*	533	50	[19]
	F: 5'- GAAAAAAAGGCTTAGAACGCCTC-3' R: 5'- GAAGATCTTTTCCGTTTTCAGC-3'	*mecC*	464		[19]
Detergent Resistance	F: 5'- GCAGAAAGTGCAGAGTTCG −3' R: 5'- CCAGTCCAATCATGCCTG −3'	*qacA/B*	361	57	[21]
	F: 5'- GCCATAAGTACTGAAGTTATTGGA −3' R: 5'- GACTACGGTTGTTAAGACTAAACCT −3'	*qacC/D* (*smr*)	195	61	[21]

25 µL, containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 1 U Taq DNA polymerase (Thermo Fisher, USA), and 2 µL of template DNA. The cycling program consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of: 95°C for 30 s, annealing for 30 s at 48°C, 50°C, 56°C, 56°C, 57°C, and 61°C for *mecA, mecC, mupA, mupB, qacA/B*, and *SMR* genes, respectively, and extension at 72°C for 45 s, with a final extension at 72°C for 7 min. PCR products were analyzed on 1% agarose gel containing a safe stain and visualized under UV light. Positive and negative controls were included in each run. The primers used are listed in Table 1.

Evaluation of Biofilm Formation Capacity

The biofilm-forming capacity of *S. aureus* isolates was evaluated using the standard microtiter plate assay. Fresh, pure colonies grown on TSA were suspended in Tryptic Soy Broth (TSB; Condalab, Spain) to achieve a turbidity equivalent to 0.5 McFarland standard ($1/5 \times 10^8$ CFU/mL). A volume of 200 µL from each bacterial suspension was dispensed into individual wells of sterile, flat-bottom 96-well polystyrene microtiter plates (Corning Inc., USA). Each strain was tested in triplicate, and plates were incubated statically at 37°C for 24 h. *Pseudomonas aeruginosa* ATCC 25923 was used as the positive control, while uninoculated TSB served as the negative control. Following incubation, biofilm quantification was performed using crystal violet staining. Planktonic cells were removed by discarding the supernatant, and wells were washed three times with phosphate-buffered saline (PBS; Gibco, USA). Biofilms were fixed with methanol for 15 minutes, then stained with 0.1% crystal violet (Sigma-Aldrich, USA) for 30 min. Excess stain was removed by triple washing with PBS, and bound dye was solubilized by adding 250 µL of 33% acetic acid to each well. After gentle shaking for 5 minutes using a plate shaker (IKA, Germany), OD was measured at 570 nm. Biofilm formation was classified based on the following criteria.[24]

OD_{NC} = Average OD of Negative Control + (3 × SD of Negative Control (NC)),

OD_{Sample} = Average OD of a Strain – OD_{NC},

OD Sample ≤ ODnc = No Biofilm,

ODnc < $OD_{Sample} \leq 2 \times OD_{NC}$ = Weak Biofilm,

$2 \times$ ODc < $OD_{Sample} \leq 4 \times OD_{NC}$ = Moderate Biofilm,

$4 \times$ ODc < OD_{Sample} = Strong Biofilm

Determining the MIC and MBC of Ethanol, Chlorhexidine, and EDTA by Broth Microdilution Method

To evaluate the antimicrobial efficacy of chlorhexidine (0.06%), 70% ethanol, and their synergistic interaction with tetrasodium EDTA35%, a microdilution assay was performed according to CLSI guidelines. *S. aureus* strains were cultured on BHI and incubated at 35 ± 2°C for 16–18 h. Two to three colonies from fresh cultures were inoculated into TSB and incubated overnight under the same conditions. The OD of each suspension was adjusted to 0.08–0.13 at 600 nm using a spectrophotometer (Biotech Instruments, USA), corresponding to approximately 10^6 CFU/mL after 1:100 dilution, respectively. For MIC determination, 100 µL of bacterial suspension was added to each well of a sterile 96-well microtiter plate. Serial dilutions of chlorhexidine, ethanol, and EDTA were prepared across columns, while rows represented different *S. aureus* strains, giving a final volume of 200 µL per well and halving the disinfectant concentration. The resulting concentration ranges tested were: ethanol, 35% to 0.068% (v/v); EDTA, 17.5% to 0.034% (w/v); and chlorhexidine, 1% (10,000 µg/mL) to 0.0019% (19 µg/mL). Column 11 (MHB + inoculum, without antimicrobial) served as the positive growth control, and column 12 (MHB + antimicrobial, without inoculum) was used as the sterility control. Plates were incubated statically at 35 ± 2°C for 18–24 h. The MIC was defined as the lowest concentration showing no visible turbidity, provided that the negative control remained clear, and the positive control showed bacterial growth. *S. aureus* ATCC 25923 was used as a reference strain.

To determine the minimum bactericidal concentration (MBC), 100 µL from wells showing no turbidity was aseptically plated onto MHA and spread using a bent Pasteur pipette. Plates were incubated at 35 ± 2°C for 18–24 h. The MBC was defined as the lowest concentration resulting in ≥99.99% bacterial killing. Synergistic interactions between

chlorhexidine or ethanol and EDTA were assessed using the checkerboard method. Serial dilutions of each agent were combined with bacterial suspensions (10^8 CFU/mL) in microtiter plates and incubated at 35 ± 2°C for 18–24 h.

Investigating the Synergistic Effects of Disinfectant Combinations Using Checkerboard Dilution Method

For determining the synergistic effect of Chlorhexidine, an EDTA checkerboard method was used.[24] Briefly, two-fold serial dilutions of each agent were prepared in microtiter plates, and the combinations were tested against bacterial isolates. After incubation, bacterial growth was evaluated, and the fractional inhibitory concentration index (FICI) was calculated to determine synergy. About 80 µL of sterile physiological serum (0.9% NaCl; Merck, Germany) was added from well 1 to well 10. In the second step, 80 µL of 0.13% EDTA was added to the first well under completely sterile conditions. The volume of the first well became 160 µL (80 µL of sterile physiological serum + 80 µL of EDTA). After complete mixing, we remove 80 µL of the contents of the first well and add it to well number 2, and this process continues until well number 10. At the end, 80 µL were removed from well number 10 and discarded. About 80 µL of 0.002% Chlorhexidine concentration is added to each well, and finally, the bacterial suspension (0.5 McFarland diluted 1 to 10) is added to all the wells in the amount of 80 µL, and the final volume of each well is reached 240 µL (80 µL of 0.5 McFarland bacterial suspension + 80 µL of Chlorhexidine + 80 µL of diluted EDTA). The fractional inhibitory concentration (FIC) index was calculated to interpret interactions, with FIC ≤ 0.5 indicating synergism, FIC > 4 antagonism, and 0.5–4 additive or indifferent effects. The fractional microbial concentration index (FMCI) represents the combined inhibitory effect of two antimicrobial agents, with lower values indicating stronger synergy and higher values suggesting antagonism.[25]

Scanning Electron Microscopy (SEM)

To further investigate the synergistic effect of EDTA and chlorhexidine on biofilm architecture, scanning electron microscopy (SEM) was employed. The most effective concentrations of both agents, determined by their ability to significantly reduce viable cell counts, were selected for imaging. Bacterial suspensions containing the selected concentrations were transferred to sterile 3 cm cell culture plates (Nunc, Denmark) and incubated for 24 h to allow biofilm formation. After incubation, the supernatant was removed, and biofilms were washed three times with PBS (pH 7.4). Samples were fixed with 2% glutaraldehyde (Sigma-Aldrich, USA) for 15 min, followed by graded ethanol dehydration (30%, 50%, 70%, 90%, and 100%) for 10 min each. Critical point drying was performed using liquid CO_2, and samples were sputter-coated with platinum (~1 nm thickness) to prevent surface charging. Biofilm morphology and structural integrity were examined using a field-emission scanning electron microscope (FE-SEM; Tescan Mira3, Czech Republic).[26]

Statistical Analysis

Data were analyzed using SPSS software, version 20 (SPSS, Chicago, IL). Descriptive statistics, along with Fisher's exact test and Pearson's chi-square test, were employed to assess differences relative to categorical variables. Continuous variables with approximately normal distributions were evaluated using Student's *t*-test, and a P-value <0.05 was deemed statistically significant.

Results

Description of Clinical Isolates

Among the 400 collected samples, 52 samples (13.0%) showed no bacterial growth, while 348 samples (87.0%) yielded bacterial isolates, comprising both Gram-positive and Gram-negative species. In this study, Gram-positive bacteria were further analyzed, among which 70 isolates were identified as *Staphylococcus aureus*, representing a prevalence of 17.5% among all 400 collected samples. Among the 70 *Staphylococcus aureus* isolates, 33 (47.1%) were obtained from hemodialysis patients, 25 (35.7%) from healthcare personnel, and 12 (17.1%) from hospital environmental samples. The highest proportion of samples was from nasal swabs, and the detailed distribution of samples is shown in Table 2.

Table 2 Characteristics of the Study Population

	Sex		Sampling Site				Hospital Wards			
	Male	Female	Nasal Cavity	Fingernails	Patient Beds	Dialysis Machines	Dialysis	Internal	Surgery	CCU
No. (%)	35 (50%)	35 (50%)	37 (52.9%)	21 (30%)	11 (15.7%)	1 (1.4%)	61 (87.1%)	7 (10%)	1 (1.4%)	1 (1.4%)

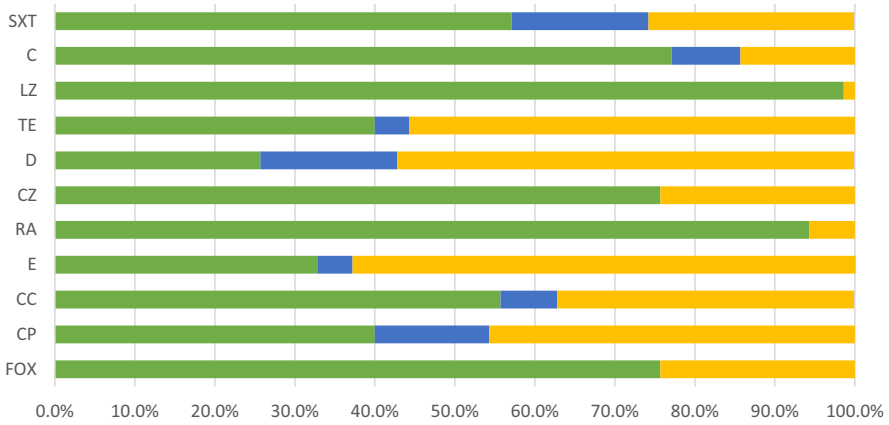
Antibiotic Susceptibility Test (AST)

As shown in Figure 1, the highest resistance among the 70 confirmed *S. aureus* isolates was observed against Erythromycin (E), Doxycycline (D), Tetracycline (Te), Ciprofloxacin (Cip), Clindamycin (CC), and trimethoprim-sulfamethoxazole (SXT). In contrast, the lowest resistance was seen against Linezolid (Lz), Mupirocin (Mup), Rifampin (RA), and Chloramphenicol (C). These findings indicate that Rifampin, Mupirocin, Linezolid, and Chloramphenicol were the most effective agents, whereas Erythromycin, Tetracycline, Doxycycline, and Ciprofloxacin demonstrated the weakest activity against the tested *S. aureus* strains. Cefoxitin (30 µg) showed that 53 isolates (75.7%) were sensitive to this antibiotic (MSSA). In contrast, 17 isolates (24.3%) were resistant to cefoxitin (MRSA). Molecular analysis of the 17 phenotypically identified MRSA isolates confirmed that all 17 (100%) carried the *mecA* gene, while 16 of them (94.1%) were also positive for the *mecC* gene.

Among the 70 *S. aureus* isolates tested by E-test, 66 (94.3%) were susceptible to mupirocin with MIC values ≤4 µg/mL, while 4 (5.7%) exhibited resistance. According to the CLSI 2025 interpretive criteria, one isolate (1.4%) showed low-level resistance with a MIC of 8 µg/mL, and three isolates (4.2%) demonstrated high-level resistance with MICs ≥512 µg/mL. Molecular analysis revealed that three of the resistant isolates harbored the *mupA* gene, producing a distinct 456 bp amplicon, confirming its role in mediating high-level resistance. The fourth resistant isolate lacked both *mupA* and *mupB* genes, suggesting the possibility of alternative resistance mechanisms. Among the 70 *S. aureus* isolates, 48 (68.6%) were classified as MDR.

Biofilm Formation Capacity

Among the tested isolates, 90% (n = 63) demonstrated strong biofilm formation, while only 10% (n = 7) exhibited moderate biofilm production. Specifically, strong biofilm formation was observed in 85.7% of fingernail isolates, 89.2% of nasal isolates, and 100% of isolates from bed surfaces and dialysis equipment. Moderate biofilm production was limited to a small fraction of isolates, ranging from 0% to 14.3% depending on the sampling site. Statistical analysis indicated no significant difference in biofilm-forming capacity among isolates from different anatomical or environmental sources (p = 0.617), suggesting a uniformly high biofilm phenotype across the studied population.



	FOX	CP	CC	E	RA	CZ	D	TE	LZ	C	SXT
Sensitive	75.7%	40.0%	55.7%	32.9%	94.3%	75.7%	25.7%	40.0%	98.6%	77.1%	57.1%
Intermediate	0.0%	14.3%	7.1%	4.3%	0.0%	0.0%	17.1%	4.3%	0.0%	8.6%	17.1%
Resistance	24.3%	45.7%	37.1%	62.9%	5.7%	24.3%	57.1%	55.7%	1.4%	14.3%	25.7%

Figure 1 Antibiotic susceptibility profiles of 70 *S. aureus* isolates tested against eight commonly used antibiotics.
Abbreviations: SXT, trimethoprim-sulfamethoxazole; C, chloramphenicol; LZ, linezolid; TE, tetracycline; CZ, cefazolin; D, daptomycin; RA, rifampicin; E, erythromycin; CC, clindamycin; CP, ciprofloxacin; FOX, cefoxitin.

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Antimicrobial Effects of EDTA, Chlorhexidine, and Ethanol

Ethanol at 8.75% eliminated 87.1% of isolates, whereas 17.5% achieved complete bactericidal activity (100%), corresponding to MBC_{50} and $MBC_{90}/_{100}$ values, respectively. Chlorhexidine exhibited uniform MIC values of 0.002% (20µg/mL), indicating consistent inhibitory activity across all isolates. At 0.002% (20µg/mL), chlorhexidine eliminated 77.1% of isolates, while 0.004% (40µg/mL) achieved complete bactericidal activity, defining MBC_{50} and $MBC_{90}/_{100}$ thresholds (Table 3). Also, molecular screening revealed that none of the 70 clinical isolates carried the *qacA/B* or *smr* genes, suggesting the absence of common disinfectant resistance determinants in this collection.

Checkerboard assays revealed that EDTA alone had MIC values 0.13%, while chlorhexidine maintained a fixed MIC of 0.0002% in all combinations. Despite these low MIC values, EDTA combined with ethanol failed to inhibit bacterial growth, suggesting a lack of synergistic interaction between these agents. In contrast, combinations of EDTA and chlorhexidine demonstrated enhanced biofilm removal capacity, ranging from 91% to 94%, with a mean efficacy of 92.71 ± 1.49%, indicating strong antibiofilm activity. Furthermore, both MIC and MBC values for EDTA alone were consistently 0.13%, reflecting uniform antimicrobial potency. These results suggest that chlorhexidine and EDTA exhibit more consistent and potent antimicrobial effects compared to ethanol, particularly in disrupting biofilm structures. The data support the potential utility of EDTA–chlorhexidine combinations in clinical disinfection protocols targeting biofilm-associated *S. aureus* infections (Table 4).

Table 3 MIC and MBC of Ethanol, EDTA, and Chlorhexidine Against Study Isolates at Different Serial Dilutions

Serial Dilution	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Ethanol 70%	35.00%	17.5%	8.75%	4.38%	2.19%	1.09%	0.55%	0.27%	0.14%	0.07%
MIC	*_	_	(70)100%	_	_	_	_	_	_	_
MBC	_	(9)12.9%	(61)87.1%	_	_	_	_	_	_	_
EDTA 35%	17.50%	8.75%	4.38%	2.19%	1.09%	0.55%	0.27%	0.13%	0.06%	0.03%
MIC	_	_	_	_	_	_	_	(70)100%	_	_
MBC	_	_	_	_	_	_	_	(70)100%	_	_
Chlorhexidine 600 µg/mL	300 µg/mL	200 µg/mL	100 µg/mL	90 µg/mL	40 µg/mL	20 µg/mL	10 µg/mL	5 µg/mL	2.5 µg/mL	1.25 µg/mL
0.06%	0.03%	0.02%	0.01%	0.009%	0.004%	0.002%	0.001%	0.0005%	0.00025%	0.000125%
MIC	_	_	_	_	_	(70)100%	_	_	_	_
MBC	_	_	_	_	(16)22.9%	(54)77.1%	_	_	_	_

Notes: *The cells (–) means is not MIC and MBC for any of isolates.

Table 4 Dilutions and Effective Ingredients of Disinfectants in the Present Study & Concentrations of Ethanol and EDTA Mixed in Each Well to Determine FICI/FMCI

Agent/Combination	MIC_{90} (%)	MBC_{90} (%)	% Isolates Killed ($MBC_{90–100}$)	*FICI/FMCI	Notes/Biofilm efficacy
Ethanol	8.75%	17.5%	100%	–	–
Chlorhexidine	0.002%	0.004%	100%	–	Uniform MIC across isolates
EDTA	0.13%	0.13%	100%	–	MIC low; MBC uniform
EDTA + Ethanol	–	–	–	indifference	No growth inhibition
EDTA+Chlorhexidine	0.0002% (CHX) 0.0005% (EDTA)	0.0002% (CHX) 0.0005% (EDTA)	100%	FIC ≤ 0.5/**FMCI low	Strong biofilm removal (91–94%, mean 92.71 ± 1.49%)

Notes: *FICI (Fractional Inhibitory Concentration Index) was calculated as follows: FICI = (MIC of agent A in combination/MIC of agent A alone) + (MIC of agent B in combination/MIC of agent B alone). The interaction was interpreted as synergy (FICI ≤ 0.5), indifferent (1 < FICI ≤ 4), or antagonism (FICI > 4). **FMCI was classified qualitatively as low, indicating a strong synergistic effect, where the combined concentrations required for inhibition were markedly lower than those of each agent alone.

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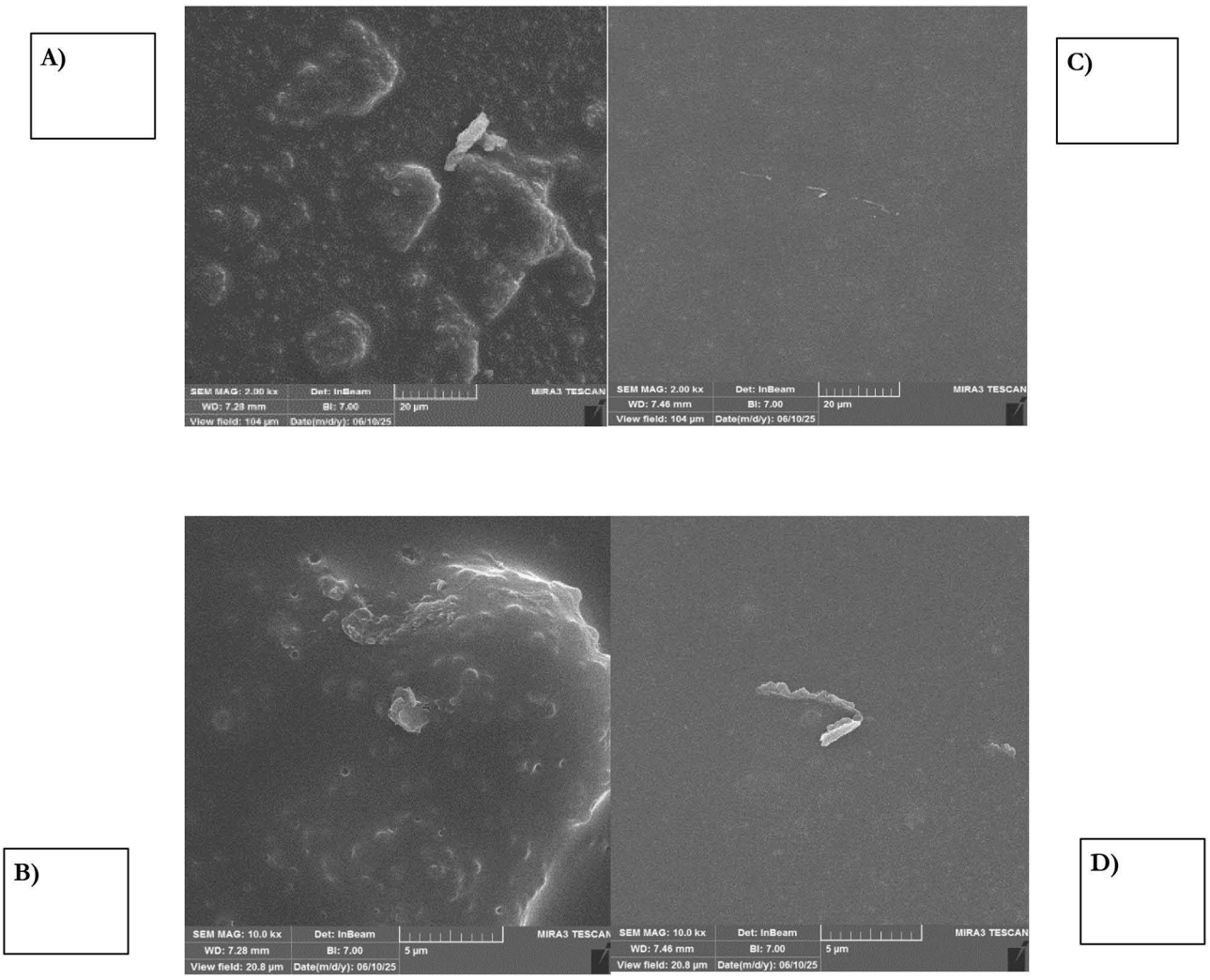

Figure 2 SEM images of *S. aureus* biofilm under different treatments. (**A**) Overview of the control biofilm, prior to exposure to antimicrobial agents, illustrating its overall structure (Magnification: 2000x, Scale bar: 20 µm). (**B**) Magnified view of the control biofilm, clearly showing more details of the cellular structure and extracellular matrix (Magnification: 10,000x, Scale bar: 5 µm). (**C**) Biofilm after exposure to 0.0002% Chlorhexidine and 0.0005% EDTA, showing complete eradication of the biofilm with negligible residual cellular debris (Magnification: 2000x, Scale bar: 20 µm). (**D**) Biofilm after exposure to 0.0002% Chlorhexidine and 0.000001% EDTA, demonstrating complete eradication of the biofilm with negligible residual cellular debris (Magnification: 10,000x, Scale bar: 5 µm).

SEM Analysis of Biofilm Disruption by EDTA and Chlorhexidine

SEM was employed to evaluate the structural integrity of *S. aureus* biofilms following exposure to selected concentrations of EDTA and chlorhexidine (Figure 2). The untreated control cells exhibited dense, multilayered biofilm architecture with intact bacterial morphology, indicative of a mature and stable biofilm. In contrast, SEM images of treated samples revealed significant disruption of the biofilm matrix. Exposure to 0.0005% EDTA combined with 0.0002% chlorhexidine resulted in visible degradation of the extracellular polymeric substance (EPS), along with morphological alterations and partial lysis of bacterial cells. A similar pattern was observed at lower concentrations (0.000001% EDTA + 0.0002% chlorhexidine), albeit with slightly reduced intensity of structural damage. These findings confirm the synergistic antibiofilm activity of EDTA and chlorhexidine, suggesting their effective penetration into the biofilm and subsequent bacterial inactivation.

Discussion

This study provides important insights into the colonization, antimicrobial resistance, and biofilm-forming ability of *S. aureus* isolates recovered from dialysis-associated hospital environments. A notably high prevalence of nasal (52.9%)

and fingernail (30%) carriage was observed, underscoring the dual role of both patients and healthcare personnel as major reservoirs for potential transmission. Such findings emphasize the importance of continuous screening, strict adherence to hand hygiene, and effective decolonization measures, particularly in dialysis units where repeated contact with vascular access and contaminated surfaces can facilitate the spread of *S. aureus*. Persistent carriage among healthcare staff can act as a constant source of contamination, highlighting the need for regular infection control audits and reinforcement of hygiene practices. In a hemodialysis unit study, the implementation of strict infection-control measures and a targeted decolonization protocol (intranasal mupirocin) led to a remarkable reduction in *S. aureus* carriage rates among patients and staff.[27] Antibiotic susceptibility testing revealed considerable resistance to erythromycin, tetracycline, and doxycycline, while rifampin and chloramphenicol remained highly effective. These results align closely with those of Shamakhteh et al, 2021,[28] who reported a ciprofloxacin resistance rate of 48% among clinical *S. aureus* isolates in Iran, and with findings by Sun et al (2020) in China,[29] where tetracycline resistance exceeded 50% in hospital-acquired strains. Such similarities suggest that antibiotic selection pressure and misuse may contribute to the persistence of resistant clones across healthcare settings. However, the resistance rates observed here contrast with reports from Northern Europe (Larsen et al, 2004),[30] where ciprofloxacin resistance was below 20%, likely reflecting stricter antibiotic stewardship programs. Despite these differences, the high susceptibility to rifampin and chloramphenicol in our study indicates that certain antibiotics retain therapeutic potential when used appropriately though rifampin should not be used as monotherapy due to the risk of rapid resistance development. In the current study, a low but clinically significant level of mupirocin resistance (4.2%) was detected, consistent with previous reports from Egypt (Taha et al, 2022)[31] and South Korea (Lee et al, 2011).[32] The presence of the *mupA* gene in most resistant isolates confirms its central role in high-level mupirocin resistance, as previously described by Abdulgader et al 2020,[33] The absence of *mupB* among our isolates aligns with findings by Goudarzi et al (2020), who reported that *mupB* is rare in clinical *S. aureus*.[34] A major finding of this study was the remarkable biofilm-forming ability of *S. aureus*, with 90% of isolates classified as strong biofilm producers. These findings indicate that biofilm formation represents an intrinsic virulence trait in dialysis environments rather than a site-specific characteristic. Biofilm formation enhances bacterial persistence, antibiotic tolerance, and immune evasion, thereby increasing the risk of recurrent infections in hemodialysis patients. Our observation is consistent with Beenken and Smeltzer (2025), who highlighted that nasal colonization by *S. aureus* serves as a major source of biofilm-associated infections, especially in individuals with indwelling medical devices.[35] Furthermore, although the isolation rate from dialysis machines was low (1.1%), this likely reflects improved disinfection practices or sampling timing rather than the true absence of contamination. As previously discussed by Beenken and Smeltzer, 2025,[35] biofilm detachment can occur intermittently, emphasizing the need for routine environmental monitoring to prevent sporadic outbreaks. The most significant outcome of this study was the demonstration of a strong synergistic anti-biofilm effect between EDTA and chlorhexidine, which achieved an average biofilm removal efficacy of 92.7%, confirmed by both checkerboard assays and SEM. Mechanistically, EDTA acts as a chelating agent that disrupts divalent cation bridges within the extracellular polymeric substance, destabilizing the biofilm matrix and facilitating deeper chlorhexidine penetration. This synergism highlights the potential of combining a matrix-disrupting agent with a conventional disinfectant to enhance anti-biofilm efficacy. Similar findings were reported by Mohamed et al 2025,[36] and Sivaranjani et al, 2021,[12] who observed that chelating agents such as EDTA significantly potentiate the activity of chlorhexidine and ethanol against resistant *S. aureus* biofilms. In contrast, ethanol alone exhibited limited anti-biofilm activity, likely due to its volatility and poor diffusion through the biofilm matrix. While the observed synergy suggests potential relevance for biofilm control strategies, further in vivo and clinical investigations are necessary before any therapeutic implications can be considered.

The absence of *qacA/B* and *smr* genes in our isolates may explain their high susceptibility to cationic biocides such as chlorhexidine, consistent with the results of Goudarzi et al 2020,[34] EDTA, through chelation of divalent cations and disruption of the biofilm matrix, potentiates the activity of antiseptics (eg., chlorhexidine and Tris-EDTA formulations) and, in in-vitro studies by Nikkhahi et al, the EDTA–chlorhexidine combinations effectively inactivated the clinical MDR strains tested (including *Klebsiella* spp. and *Escherichia coli*).[37]

From a practical infection control standpoint, these findings have several implications. First, the preparation and application of disinfectants at optimal concentrations are essential to ensure maximal antimicrobial activity and to avoid

the emergence of biocide tolerance. Second, continuous education of healthcare personnel and adherence to disinfection protocols are crucial to minimize cross-contamination. Third, incorporating synergistic disinfectant combinations such as EDTA and chlorhexidine into routine cleaning regimens, particularly for high-touch surfaces and dialysis-related equipment, could significantly improve biofilm eradication and reduce environmental contamination. Similar strategies have been proposed by Lavoie et al, 2025,[38] who emphasized that biofilm-targeted disinfection protocols are essential in preventing nosocomial transmission in dialysis environments. This study has several limitations. First, the sample size was relatively limited and derived from a restricted geographic region, which may affect the generalizability of the findings. Second, the antibiofilm experiments were conducted exclusively under in vitro conditions, which may not fully replicate the complexity of in vivo environments. Consequently, the translational applicability and clinical relevance of these findings remain to be validated in more complex biological systems.

Taken together, this study highlights the complex interplay between antibiotic resistance, biofilm formation, and disinfectant efficacy in hospital-associated *S. aureus* isolates. The findings provide mechanistic insight into potential strategies that could inform future approaches to controlling biofilm-mediated persistence and transmission in healthcare environments. Future research may focus on exploring additional synergistic combinations of disinfectants, investigating their effects on biofilm-related gene expression, and evaluating their safety, efficacy, and feasibility in more complex in vivo or clinical models.

Conclusion

The current study emphasizes the coexistence of mupirocin resistance, multidrug resistance (MDR), and robust biofilm-forming capacity among carrier *S. aureus* isolates in dialysis-associated hospital environments. The consistently strong biofilm-producing phenotype identified across anatomical and environmental sources highlights the persistence potential of these strains and their possible role in nosocomial transmission.

The observed in vitro synergistic antibiofilm interaction between EDTA and chlorhexidine provides mechanistic insight into matrix disruption strategies that may enhance disinfectant penetration. Nevertheless, as these findings are derived from controlled laboratory models, they should be interpreted with caution. Further in vivo and clinical investigations are required to validate the safety, efficacy, and translational applicability of such approaches in healthcare settings. Overall, these findings reinforce the importance of continuous resistance surveillance and strict infection control measures in high-risk hospital environments.

Data Sharing Statement

The datasets generated and analyzed during the current study are not publicly available due to privacy or ethical restrictions but are available from the corresponding author on reasonable request.

Ethical Approval

All methods in the current study were performed in accordance with the guidelines of the Ethics Committee of Qazvin University of Medical Sciences, with approval number 1402/171 IR.QUMS.REC. And all procedures were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to sample collection.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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