



# Microbes and Infectious Diseases

Journal homepage: <https://mid.journals.ekb.eg/>

## Original article

### Evaluation of chlorhexidine and povidone iodine activity against biofilm forming *Staphylococcus epidermidis* clinical isolates

Amal Fathalla Makled <sup>1</sup>, Mohamed Medhat Ghareib <sup>2</sup>, Sahar Ali <sup>1</sup>, Mostafa Mahmoud Sharweed <sup>\*2</sup>, Asmaa shaban Sleem <sup>1</sup>

1- Medical Microbiology and Immunology Department, Faculty of Medicine, Menoufia University, Shebin El-kom32512Egypt

2- Botany and Microbiology Department, Faculty of Science, Menoufia University, Shebin El-kom32512, Menoufia, Egypt

#### ARTICLE INFO

##### Article history:

Received 4 October 2023

Received in revised form 16 December 2023

Accepted 27 December 2023

##### Keywords:

*S. epidermidis*  
Biofilm  
Resistance  
Chlorhexidine  
Povidone iodine

#### ABSTRACT

**Background:** *Staphylococcus epidermidis* (*S. epidermidis*) has emerged as a major etiological factor in implant-related infections, primarily due to its biofilm forming ability. These biofilms enhances bacterial resistance to antimicrobial agents. **Aim:** This study aimed to detect and characterize drug-resistant *S. epidermidis* strains in clinical samples obtained from Menoufia University Hospitals, with evaluating their biofilm-forming ability and assessing the antimicrobial activity of chlorhexidine and povidone iodine. **Methodology:** Drug-resistant *S. epidermidis* strains were identified using the Vitek2 system and their biofilm producing ability was determined. The presence of biofilm-related genes (*icaA* and *icaD*) was confirmed through polymerase chain reaction (PCR). Multi-drug resistant *S. epidermidis* isolates capable of biofilm formation were exposed to varying concentrations of chlorhexidine (0.025%, 0.035%, 0.05%, 0.12%) and povidone iodine (1.5%, 3.5%, 7.5%, 10%) for different exposure times. **Results:** Of 354 clinical isolates, 75 (21.2%) were identified as drug-resistant *S. epidermidis* using Vitek2 system. Biofilm production was observed and confirmed by the presence of *icaA* / *icaD* genes among 61-isolates. Chlorhexidine demonstrated significant effectiveness in vitro at concentration of 0.05% with a short exposure time of 1 minute. In contrast, povidone iodine required higher concentrations and prolonged exposure times to exhibit antibacterial activity. **Conclusion:** Chlorhexidine was an effective antimicrobial agent against *S. epidermidis*, particularly when used at clinically available concentrations (0.05%) with short exposure time, whereas povidone-iodine required higher concentrations with prolonged exposure times. Further investigations are warranted to optimize the use of these antiseptics.

#### Introduction

*Staphylococcus epidermidis* is a common opportunistic nosocomial pathogen. It is responsible for antimicrobial resistant infections due to its

ability to form biofilms in tissues where bacterial cells enter in a self-produced extracellular matrix in an aggregate fashion with strong intercellular contact creating a barrier shields microbial cells from antibiotics, antiseptics, disinfectants and host

DOI: 10.21608/MID.2023.240761.1631

\* Corresponding author: Mostafa Mahmoud Sharweed

E-mail address: amalmakled@msn.com

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immune system [1]. Biofilm formation is under the control of intracellular adhesion (*ica*) gene products. Among *ica* genes, *icaA* and *icaD* are major biofilm constructing genes; enhancing cell to cell adhesion with abundant extracellular matrix expression [2].

The pandemic spread of antibiotic resistance enlightens the management of hospital acquired infections as a difficult infection control challenge. Control and preventive measures are widely focusing on; hand washing, well planned antibiotic stewardship programs and antiseptic skin cleansers [3].

Antiseptic agents have reported antibacterial activity with broader spectrums and reduced chance for emergence of bacterial resistance, relative to antibiotics. As a result, antiseptics are considered as excellent substitute to antibiotics for the prevention and treatment of hospital acquired multidrug resistant bacterial infections [4].

Both chlorhexidine and povidone iodine antiseptics are over the counter since 1950. They are available on the World Health Organization's list of essential medicines as being recommended for eradication and control of nosocomial bacteria. They are globally applied antiseptic agents with broad spectrum efficacy against both Gram-positive and Gram-negative bacteria [5].

Chlorhexidine has been shown to decrease microbial flora on the skin through bacterial cell wall disruption. Consequently, they are used to prevent bacterial colonization following invasive procedures. Povidone-iodine has been broadly applied in medicine as pre- and post-operative skin cleanser treating and preventing wound infections through destructive oxidation of essential bacterial components [6,7].

Here, we aimed to detect *Staphylococcus epidermidis* in Menoufia university hospitals, study its antimicrobial profile, evaluate its biofilm producing ability relative to drug resistance, and assess chlorhexidine and povidone iodine activity against biofilm producing drug resistant isolates to highlight their roles in control of *S. epidermidis* nosocomial infections.

### Patients and methods

This cross-sectional study involved the analysis of seventy-five *S. epidermidis* isolates collected from 354 different clinical samples over a duration of seven months. Written consents, as well as comprehensive personal and clinical histories, were

gathered from various departments and intensive care units (ICUs) of Menoufia University Hospitals. The study protocol received approval from the local Ethics Committee of Menoufia University (IRB No 8/2023 MICR 7).

### Bacterial identification

The clinical specimens involved: blood, urine, sputum, pus and wound swabs were promptly delivered to the Medical Microbiology and Immunology laboratory at the Faculty of Medicine, Menoufia University, for examination. Each specimen was aerobically cultivated and incubated for 48 hours on nutrient, MacConkey, human blood, and mannitol salt agar. All isolates were identified using standard diagnostic procedures with semi-quantitative assay to exclude contamination [8]. Species confirmation and antimicrobial susceptibility testing for all *S. epidermidis* strains were automatically reported by the VITEK 2 compact system (bioMérieux, France) in the Medical Microbiology and Immunology Department at the National Liver Institute, Menoufia University.

### Biofilm detection

Multi-drug resistant *S. epidermidis* strains (non-susceptible to at least one agent in three or more antimicrobial categories) underwent further testing for biofilm formation. Phenotypic testing was performed using microtiter plate method, followed by genotypic confirmation through the detection of *icaA* and *icaD* biofilm-associated genes.

**Microtitre plate method:** A sterile 96-well microtiter plate (Catalog number: KG10096, Korea) was inoculated with diluted *S. epidermidis* cultures in 1% glucose supplemented trypticase soy broth (TSBG). After 48 hours of aerobic incubation at 37°C, plate contents were discarded and washed twice with phosphate buffered saline. To stain the biofilm, crystal violet was added. The microtiter plate was washed again and examined for remaining violet discoloration, indicating biofilm formation. Then stained biofilms were solubilized with ethanol. The optical density of the plate contents was measured at 490 nm. Triplicate testing was performed for each strain, and standard deviations of the readings were calculated [9,10].

### Detection of biofilm genes (*icaA*,*icaD*)

Bacterial DNA was extracted using Easypure DNA extraction kit (cat. no. EE161) following manufacturer's instruction. Extracted DNA were preserved at -80°C after assessment using the

NanoDrop™ 2000 system (Thermo Scientific, USA). Conventional PCR steps included initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1 min with a final extension at 72°C for 2 min. Used *icaA* primer sequence was **F: 5'TCTCTTGCAGGAGCAATCAA 3'-R: 5'TCAGGCACTAACATCCAGCA 3'** while *icaD* used sequence was **F:5'ATGGTCAAGCCCAGACAGAG3'R:5'CGTGTTTTCAACATTTAATGCAA3'**. The amplified DNA bands were detected by agarose gel electrophoresis (188bp for *icaA* & 198bp for *icaD*) [11].

### Evaluation of povidone-iodine and chlorhexidine antibacterial effect

#### 1-Determination of minimum bactericidal concentrations (MBC) and optimum contact time of povidone-iodine and chlorhexidine

The broth microdilution technique was employed to detect the MBC of povidone-iodine (BETADINE® Mundi pharma Antiseptic Solution 10%) and chlorhexidine gluconate (VIRUSAN® Amity international, UK, 0.05% Solution). Each isolate was cultured overnight on mannitol salt agar, and a few colonies were suspended in Mueller-Hinton broth to achieve turbidity equal to a 0.5 McFarland standard. About 100 µl of prepared dilutions of povidone-iodine (1.5%, 3.5%, 7.5%, 10%) and chlorhexidine (0.025%, 0.035%, 0.05%) in deionized water were added to 100 µl of the bacterial suspension in each well. The microtiter plates were incubated aerobically at 37°C for 24 hours. The MBC was defined as the lowest concentration of the antiseptic that resulted in no visible bacterial growth. The optimum contact time was determined by examining the plates at different time intervals (0.5, 1, 3, 5 and 10 minutes) to be sub cultured on Muller Hinton agar plates detecting no growth which is indicative of bactericidal concentration relative to contact time [12].

#### 2-Determination of povidone-iodine and chlorhexidine role in eradication of living bacteria in established biofilms

Selected *S. epidermidis* isolates were allowed to grow in TSBG to enhance biofilm formation. After 48 h tubes contents were discarded and washed twice with PBS to remove planktonic bacterial cells. Now only biofilm forming cells were left attached to tube walls. Using sterile tips micropipette, biofilm remnants were scraped in 1.5 ml of previously

prepared biocides solutions to be tested followed by 2 min on vortex enhancing biofilm disruption. Tubes were incubated for the last time under favorable conditions to encourage growth of any living biofilm forming bacterial cells. After maximum 3 consecutive days of incubation, tubes were tested for visible turbidity indicating biocide failure while absence of turbidity indicated biocide excellence [13].

### Statistical analysis of the data

The data were analyzed using IBM SPSS software package version 20. Results were represented using numerical values and percentages. The chi-square test was employed to analyze categorical variables and assess the differences between various groups.

### Results

Microbiological analysis of the clinical samples obtained (354 in total) in current study, revealed that 140/354 (39.5%) were infected with Staphylococci, among which, 75 samples (21.18%) were identified as strains of *S. epidermidis* using the VITEK system. As depicted in **figure (1)**, the intensive care units (ICUs) were the primary department from which 42.6% (32/75) of the *S. epidermidis* isolates were detected followed by internal medicine and surgery departments. Furthermore, blood samples were the most abundant clinical samples collected for analysis by 65.3%, followed by urine. Several factors were identified as significant risk factors contributing to nosocomial infections caused by *S. epidermidis*, including old age, diabetes, prolonged hospitalization, and invasive procedures by more than 60%.

The antimicrobial susceptibility profile of *S. epidermidis* strains detected by the VITEK system as shown in **table (1)**, exhibited complete resistance to penicillin and oxacillin, with a resistance rate of 100%. Fusidic acid resistance was observed in 93.3% of the strains, followed by tetracycline (88%), erythromycin (80%), clindamycin (73.3%), ciprofloxacin (72%), and gentamycin (60%). In contrast, all isolates demonstrated susceptibility to vancomycin and linezolid. Furthermore, tigecycline, teicoplanin, and rifampicin exhibited high activity as antimicrobial agents, with sensitivity percentages of 84%, 81.3%, and 80%, respectively.

Surpassing our expectations, a high proportion of multi-drug resistance was observed among the isolates, with 65 out of 75 (86.7%) exhibiting resistance. Among these, 29 isolates (44.6%) demonstrated extensive drug resistance.

In our study, bacterial biofilm formation was a significant virulence factor contributing to drug-resistant infections by more than 91%. Here, biofilms detection was conducted using the microtiter plate gold standard method. Out of 65 isolates, 61 (93.8%) were confirmed biofilm producers, with 41 (65.6%) classified as moderate producers and 20 (34.4%) as strong biofilm producers.

In our study, we observed the presence of both-biofilm genes, *icaD* and *icaA*, in combination in 30 out of 61 samples, accounting for 49.2% of the cases. Specifically, we found *icaA* in 10 out of 61 samples (16.4%) and *icaD* in 18 out of 61 samples (29.5%), as depicted in **figures (2,3)**.

Our results were alarming as biofilm formation was significantly observed among drug resistant *S. epidermidis* isolates with 33/36 (91.7%)

among MDR isolates and 28/29 (96.5%) among XDR isolates as shown in **table (2)**.

In our study, it was determined that the least effective bactericidal concentration-contact time combinations for chlorhexidine was 0.05% for a maximum of 1 minute with efficacy exceeding 93% within a short exposure time of 1 minute .While, biofilm inhibition was recorded by 57.4% for the same concentration. Povidone-iodine demonstrated efficient bactericidal activity at concentrations of 3.5% for 10 minutes, 7.5% for 5 minutes, and 10% for 1 minute. Additionally, concentrations of 3.5%, 7.5%, and 10% of povidone-iodine were effective in eradicating *S. epidermidis* biofilms after 10-, 5-, and 1-minute exposures, respectively, with a 100% success rate observed with the 10% preparation as shown in **table (3)**.

**Table 1.** VITEK anti-biogram of *Staphylococcus epidermidis* isolates.

Antimicrobial agent	<i>S. epidermidis</i> isolates (N=75)			
	Sensitive		Resistance	
	No.	%	No.	%
Penicillin/Oxacillin	-	-	75	100
Vancomycin	75	100	-	-
Erythromycin	15	20	60	80
Tetracycline	9	12	66	88
Tigecycline	63	84	12	16
Ciprofloxacin	21	28	54	72
Moxifloxacin	58	77.3	17	22.7
Trimethoprin/ Sulfamethoxazole	55	73.3	20	26.7
Clindamycin	20	26.7	55	73.3
Rifampicin	60	80	15	20
Linezolid	75	100	-	-
Teicoplanin	61	81.3	14	18.7
Gentamycin	30	40	45	60
Fusidic Acid	5	6.7	70	93.3

**Table 2.** Biofilm production in relation to drug resistance.

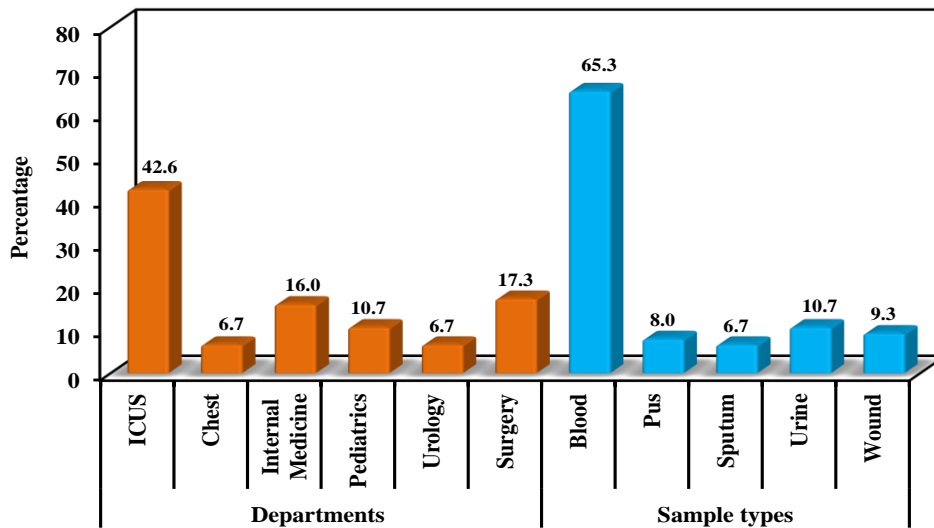
Drug resistance	Drug resistant isolates (n=65)		Biofilm production				$\chi^2$	p
			Absent (n = 4)		Present (n = 61)			
	No.	%	No.	%	No.	%		
Multi-drug (MDR)	36	55.4	3	8.3	33	91.7	0.664	0.022
Extreme-drug(XDR)	29	44.6	1	3.5	28	96.5		

**Table 3.** Efficacy of tested antiseptics concentrations against virulent *Staphylococcus epidermidis*.

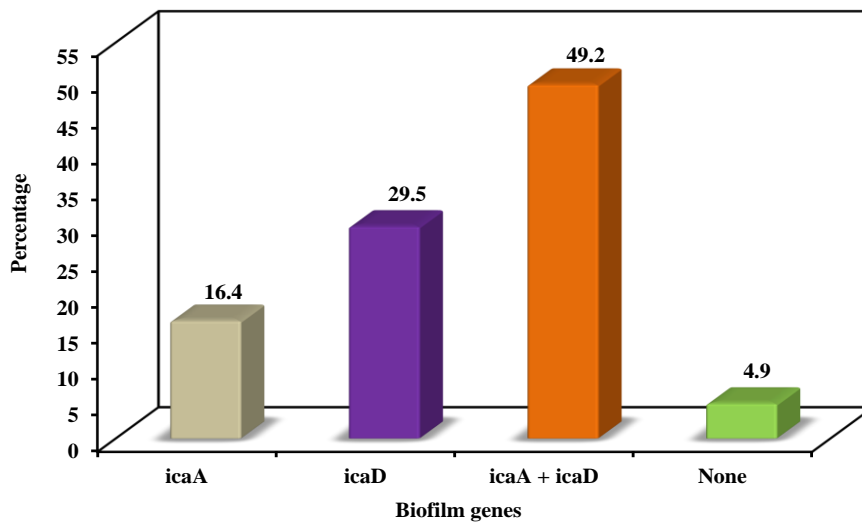
Antiseptic-solution	Tested concentration%	Least effective contact time (minutes)	<i>S. epidermidis</i> strains inhibition No.=65		<i>S. epidermidis</i> biofilm inhibition No.=61	
			No.	%	No.	%
Chlorhexidine	0.025	*ND	-	-	-	-
	0.035	**NA	8	12.3	-	-
	0.05	1	61	93.8	35	57.4
Povidone-Iodine	1.5	**NA	15	23	-	-
	3.5	10	57	87.7	28	45.9
	7.5	5	65	100	42	68.8
	10	1	65	100	61	100

\*ND= not detected \*\*NA=not applicable

**Figure 1.** Distribution of *S. epidermidis* isolates among different hospital departments and various sample types.



**Figure 2.** Analysis of *icaA* and *icaD* genes among biofilm producing isolates.

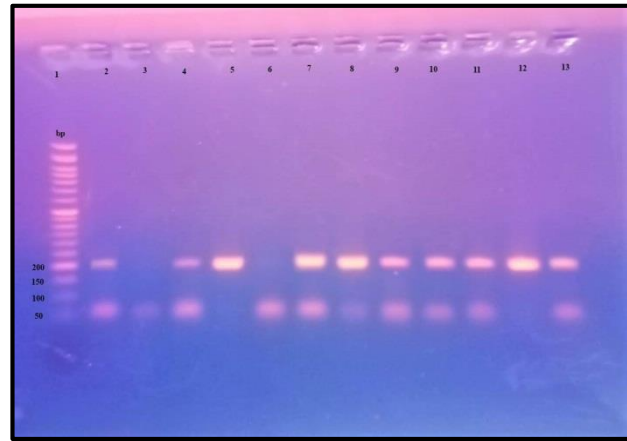


**Figure 3.** Agarose gel electrophoresis showing PCR amplified products of biofilm gene (*icaA* and *icaD*) among *S. epidermidis* virulent isolates where Lane 1 indicates DNA molecular size marker (50-1000 bp).

- A) *icaA* gene, Lanes : 5,6,7,11,12,13 were positive for the *icaA* (188 bp);  
Lanes: 2,3,4,8,9,10 were negative for the *icaA* gene
- B) *icaD* gene, Lanes: 2,4,5,7,8,9,10,11,12,13 were positive for the *icaD* (198bp);  
Lanes: 3,6 were negative for the *icaD* gene



(A)



(B)

### Discussion

Hospital-acquired infections, particularly those caused by *S. epidermidis*, are a significant problem worldwide. *Staphylococcus epidermidis* is a common bacterium found on human skin and is often associated with opportunistic infections [1,11]. According to VITEK system results in this study, *S. epidermidis* was detected by 75 /354 (21.18%) among obtained clinical isolates. This finding aligned with a recent Egyptian study that reported a 22.6% prevalence of coagulase-negative staphylococci, including *S. epidermidis* [14], as well as an Asian study conducted by **Parul et al.** in 2021 [15].

In the current study, most *Staphylococcus epidermidis* strains were significantly isolated from blood samples obtained mainly from ICU patients by 42.6% (32/75) with significant risk factors contributing to nosocomial infections predominance including; old age, diabetes, prolonged hospitalization, and invasive procedures. These findings align with reports from the Centers for Disease Control and Prevention (CDC), identifying these factors as major contributors to *S. epidermidis* infections in healthcare settings by more than 80%. [3,5, 16- 18].

It is worth noting that most ICU patients often suffer from chronic debilitating diseases and require invasive medical devices, which can

enhance bacterial colonization and increase the risk of infection [19]. Efforts should be made to implement strict infection control measures, such as proper hand hygiene, device sterilization, and surveillance programs, to mitigate the spread of *S. epidermidis* and other hospital-acquired infections in healthcare facilities especially ICUs.

In our study, all isolated *S. epidermidis* strains exhibited complete resistance to penicillin and oxacillin, with a resistance rate of 100%. On the other side almost 100% susceptibility was observed with vancomycin and linezolid, These findings aligned with previous studies conducted by **Chabi et al.** (2019) and **Nicolosi et al.** (2020). They indicated that the treatment of methicillin-resistant *S. epidermidis* (MRSE) had become increasingly challenging due to the bacterium's resistance to multiple antibiotics [20,21].

The potential for methicillin-resistant *S. epidermidis* strains to develop multi-drug resistance is becoming increasingly evident on a global scale. This finding is particularly alarming, as it is consistent with the results reported by **Eladli et al.** in 2019. Considering these findings, researchers have recommended linezolid, rifampicin, and vancomycin antibiotics as first-line therapy for device-associated staphylococcal infections, which aligns with the records of our study [14-16,22-24].

*Staphylococcus epidermidis* strains usually resist against several types of antibiotic classes such as tetracyclines, aminoglycosides, cephalosporins, fluoroquinolones, penicillins, and macrolides. Nowadays, resistant *S. epidermidis* has become a serious problem in hospitals [18]. Drug resistance is directly related to biofilm formation which is thought to aid poor antibiotic penetration, nutrition restriction and slow growth, plus the development of persister cells [25].

Bacterial biofilm has long been considered as a virulence factor contributing to infection associated with various medical devices. In our study, detection of biofilm formation of clinical isolates was carried out by microtitre plate method which is the gold standard method for biofilm detection as documented by **Nashat et al.** in 2022. Also, **Aladarose et al.** in 2019 reported also that this method is accurate and reproducible for screening and determination of biofilm production. Therefore, we used this method in current study where out of 65 isolates, 61 (93.8%) were confirmed biofilm producers, with 41 (65.6%) classified as moderate producers and 20 (34.4%) as strong biofilm producers. These findings approximate the data reported in similar studies, where moderate and strong biofilm production ranged from approximately 20% to 37% and 35% to 51%, respectively [9,20,22,26,27].

Biofilm formation is a complex process regulated by various genes, including *icaA*, *icaB*, *icaC*, *icaD*, and *icaR*. Among these genes, *icaA* and *icaD* play a significant role in biofilm production in *S. epidermidis* [20].

In our study, we observed the presence of biofilm genes, *icaD* and *icaA* by 16.4% and 29.5% respectively. An European study conducted by **Cal et al.** in 2022 and his colleagues reported approximating results, with 37% of the samples positive for *icaD* and 25% positive for *icaA* biofilm genes [28]. Additionally, **Luiza et al.** (2014) detected *icaA* in 12.5% of the samples compared to 20% for *icaD*, and only 7.7% showed the presence of both genes. These variations in detection rates can be attributed to gene mutations, particularly in *icaA*, which may explain its lower detection rate compared to *icaD* [29,30].

It is worth noting that biofilm formation can also occur through *ica*-independent mechanisms, involving several surface proteins. This could explain the presence of biofilm formation in few isolates in our current study, despite the

absence of both *icaA* and *icaD* genes [2]. These findings highlight the intricate nature of biofilm formation and the involvement of multiple genetic and molecular factors in the process.

Biofilm-forming microbes have the ability to cause various diseases, and according to Centre of Disease Control (CDC) report, biofilm-forming microbes are responsible for causing 65-80% of drug resistant infections. Our results were alarming as biofilm formation was significantly observed among drug resistant *S. epidermidis* isolates with 33/36 (91.7%) among MDR isolates and 28/29 (96.5%) among XDR isolates in compliance with multiple European and Arabian studies [1,2,5,30,31].

It is important to note that the increasing resistance of *S. epidermidis* strains to commonly used antibiotics poses a significant clinical concern. The susceptibility patterns observed in this study reflect the need for judicious antibiotic use with the implementation of appropriate antibiotic stewardship programs and strict infection prevention protocol emphasizing hand washing and optimum disinfection as the corner stone for infection control of drug resistant *S. epidermidis* infections.

Despite the widespread use of disinfectants in the healthcare system, there is a lack of comprehensive research on their antibacterial capabilities and effectiveness. However, studies have shown that chlorhexidine, a commonly used disinfectant, exhibits excellent antimicrobial properties when dissolved in water and delivered in an optimal concentration. It has been found to effectively combat various pathogens, including bacteria, yeasts, and viruses [32]. In our research, it was determined that the least effective concentration-contact time combinations for chlorhexidine were 0.05% for a maximum of 1 minute with significant biofilm destruction. Promising results have been reported regarding the anti-biofilm effect of chlorhexidine. Recent American studies [33,34] support these findings and recommend a concentration of 0.12% chlorhexidine as a chemotherapeutic agent for reducing biofilms, particularly those caused by gram-positive bacteria with larger negatively charged cells. However, a European study conducted by **Salvatico et al.** in 2019 reported that 0.5% chlorhexidine was the least effective antibacterial concentration, highlighting the need for further research [35].

In this study, we selected antiseptic concentrations commonly used in clinical practice for povidone-iodine which demonstrated efficient bactericidal activity at concentrations of 3.5% for 10 minutes, 7.5% for 5 minutes, and 10% for 1 minute. Additionally, concentrations of 3.5%, 7.5%, and 10% of povidone-iodine were effective in eradicating *S. epidermidis* biofilms after 10, 5, and 1 minute exposures, respectively, with a 100% success rate observed with the 10% preparation. These results align with current European studies [7,13,36]. However, it is worth noting that the bactericidal concentrations required for povidone-iodine were higher than those for chlorhexidine, suggesting that povidone-iodine has somewhat weaker antibacterial efficacy in vitro [37]. Lower concentrations of the tested solutions were deemed ineffective either due to impractical application (requiring contact times longer than 10 minutes) or inadequate effectiveness even with prolonged exposure. Using higher concentrations would not only be unnecessary but may also lead to unwanted side effects. In a previous study conducted by **Kenneth et al.** in 2018, chlorhexidine was found to be superior to povidone-iodine in combating staphylococci biofilm producers, with a minimum effective concentration of 2%, which is higher than the effective concentration of 0.05% tested in our study [33].

In summary, disinfectants are indispensable tools in infection control, and their optimal use at bactericidal concentrations is paramount. Our research highlights the effectiveness of chlorhexidine at a clinically available concentration, while also emphasizing the need for continued investigation into the broader spectrum of antimicrobial activity. By pursuing further studies, we can advance our practice and ensure the maximum efficacy of disinfectants in combating nosocomial infections.

### Conclusion

The use of disinfectants is crucial for effective infection control and the prevention of nosocomial infections. However, it is important to emphasize that their efficacy relies on the utilization of optimum lethal concentrations. In this study, chlorhexidine demonstrated significant effectiveness in vitro in eradicating virulent *S. epidermidis* at a clinically available concentration of 0.05% within a short exposure time of just one minute. On the other hand, povidone-iodine required

higher concentrations and prolonged exposure times to achieve similar outcomes. It is essential to conduct further studies to expand our understanding of the topic, particularly concerning the impact of these disinfectants on other types of microbes.

### Conflicts of interest

The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

### Funding

Not declared.

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