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Molecular distribution of biocide resistance genes and susceptibility to biocides among vancomycin resistant *Staphylococcus aureus* (VRSA) isolates from intensive care unit (ICU) of cardiac hospital- A first report from Pakistan

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ABSTRACT

Background: The study was conducted with the aim to investigate the VRSA isolates in terms of their susceptibility to routinely used biocides influenced by the co-occurrence of biocide resistant gene (BRGs) and efflux pumps genes.

Methodology: Frequently touched surfaces within intensive care unit (ICU) of cardiac hospital were classified into three primary sites i.e., structure, machines and miscellaneous. Over a period of six months (January 2021 to July 2021) twenty three swabs samples were collected from these sites. Subsequently, these samples underwent both phenotypic and molecular methods for VRSA isolation and identification. Susceptibility and efficacy testing of biocides (benzalkonium chloride (BAC), cetrimide (CET) and chlorhexidine gluconate (CHG)) were evaluated using microdilution broth and suspension method. Furthermore, specific primers were used for singleplex PCR targeting BRGs (*cep*A, *qac*A, and *qac*E) and efflux pump (*nor*A, *nor*B, *nor*C, *sep*A, *mep*A and *mde*A) associated genes.

Results: We found that 72.2 % *S. aureus* demonstrate the presence of *van*A or *van*B genes with no significant difference among three sites ($p > 0.05$). *cep*A is the most dominant BRGs followed by *qac*A and *qac*E from structure site as compared to other sites ($p < 0.05$). BAC showed reduced biocide susceptibility and MIC50. There was no significant difference between presence or absence of BRGs and high MIC values of VRSA isolates from all three sites. However, efflux pump genes (EFPGs) particularly *nor*A and *nor*A + *sep*A had a significant association with BRGs and reduced biocide.

Conclusion: BAC is the most effective disinfectant against VRSA. Proper and controlled use of BAC is required to overcome the VRSA contamination. We recommend continuous monitoring of the BRGs prevalence for better prevention of microorganism dissemination and infection control in hospitals.

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1. Introduction

The earliest strain of vancomycin-intermediate *Staphylococcus aureus* (VISA) was revealed in May 1, 9961 in Japan [1]. These isolates were reported globally and developed vancomycin resistance [2]. The WHO recently designated vancomycin resistant *Staphylococcus aureus* (VRSA) as a high priority pathogen that has the potential to increase fatality rates globally [3].

The resistance to vancomycin is associated with *van* genes. This could be inherited by both mutation and horizontal transfer of vancomycin-resistant genes among the bacteria. So far, 11 *van* genes, *van*A, *van*B, *van*D, *van*F, *van*I, *van*M, *van*C, *van*E, *van*G, *van*L, and *van*N, have been described [4]. Among them, *van*A and *van*B genes are the most important and frequently reported from hospital isolates [5]. Being highly resistant to VRSA is known for high virulence and pathogenicity potential. They are able to produce thick biofilm owing to their thick cell walls. This in turns makes VRSA resistant to surface disinfectants and biocides [6]. These isolates persist in the healthcare facilities and may results in dissemination of antimicrobial resistance genes (ARGs) and biocides resistant genes (BRGs) [7,8].

Disinfection is pivotal to the mitigation of nosocomial pathogen dissemination and infection control [9]. Biocides, such as antiseptics and disinfectants, have been often used in healthcare settings for long time to maintain environmental bioburden [10]. With the generations of different biocides formulations, biocide resistance is a also a substantially emerging challenge [11,12].

Biocides resistance may be an intrinsic property, or it may arise either by chromosomal gene mutation or by the acquisition of biocide resistant genes (BRGs) such as *qac*A and *cep*A. These genes may be associated with plasmids or transposons [13]. Efflux pumps also play important role in this process and result in reduce biocide susceptibility in pathogens [13,14].

Here we hypothesized, that efflux pump resistance (EFGs) mediated genes may be associated with BRGs and reduced biocide susceptibility among VRSA isolates. The study was designed with the aim to analyze the VRSA isolate from hospital surfaces for BRGs and investigate the association between BRGs and biocide susceptibility along with routinely used biocide efficacy testing. It is a pressing issue in health care settings, yet there is only a small number of studies available worldwide addressing reduced biocides susceptibility and biocide minimum inhibitory concentration (MICc) values among nosocomial pathogens.

2. Material and methods

2.1. Study design

The study was conducted over a span of six months (January 2021 to July 2021) at a tertiary level autonomous public sector hospital for cardiac diseases (Faisalabad institute of cardiology) with the capacity of 202 beds. This hospital situated in Punjab province with largest population of ≈100 million. The primary catchment area is Faisalabad with ≈3.5 million population and bears the burden of surrounding 3 district. The charcoal swab (ThermoFisher, USA) samples were collected from frequently touched surfaces

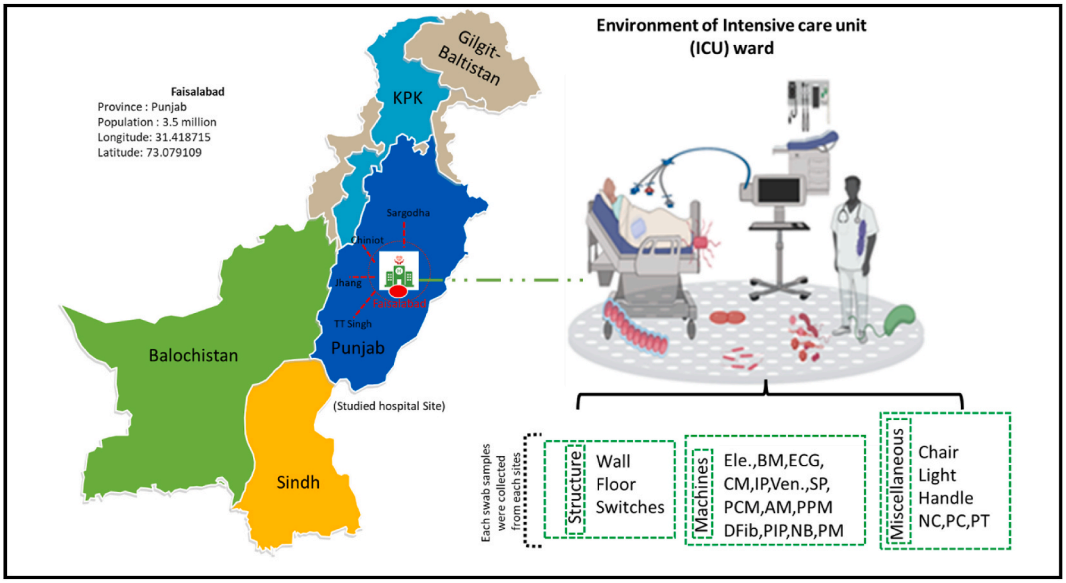

Fig. 1. Diagrammatical explanation of sample collection. Swab samples were collected from three frequently touched sites of ICU of cardiac care hospital situated in Punjab province bears the burden of surrounding three districts. For full coverage of ICU these sites were divided into 1. Structure (walls, floor, and switches), 2. Machines [Ele. (elevator), BM (biometric machine), ECG (electrocardiogram), CM (cardiac monitor), Ven (ventilator), IP (infusion pump), SP (spirometer), PCM (paeds cardiac monitor), AM (anesthesia machine), PPM (permanent pacemaker), DFiB (D fibrillation machine), PIP (paeds infusion pump), NB (nebulizer) and PM (pacemaker)], 3. Miscellaneous [chairs, lights, doorknobs, NC (nursing counter), PC (pharmacy counter) and PT (paeds table)].

of intensive care unit (ICU) ward by selecting the 10 and 5 cm^2 areas according to standard operating procedure of environmental sampling Fig. 1 [15].

2.2. Bacterial strains

These samples were streaked on selective agar (Mannitol salt agar, Oxoid, UK) for *S. aureus* isolation. Briefly, all suspected isolates from selective agar were gram stained and tested for catalase (bioMerieux, Marcy L'Etoile, France), coagulase (Remel™ Coagulase Plasma - ThermoFisher Scientific, UK) and latex agglutination test (Slidex Staph Plus, bioMerieux, Marcy L'Etoile, France) [16].

2.3. Determination of vancomycin resistant Staphylococcus aureus (VRSA)

All confirmed *S. aureus* isolates underwent antibiotic susceptibility testing using disc diffusion method based on Clinical and Laboratory Standards Institute (CLSI) guidelines [17]. The phenotypically confirmed VRSA isolates were subjected for genomic DNA purification (ThermoFisher, USA). confirmation of VRSA was conducted using singleplex polymerase chain reaction (PCR) with specific oligonucleotide primer sequence and AccuPrime master mix (Invitrogen, USA) (Supplementary Table 1a). Applied Biosystems (ThermoFisher, USA) thermocycler was programmed with the initial denaturation, (10 min at 94 °C; 30 cycles with a 30 s denaturation step at 94 °C), a 45 s annealing step at 50 °C and a 30 s extension step at 72 °C and 10 min extension step at 72 °C and a holding step at 4 °C [16]. Resulting PCR product were electrophoresed, stained (10 μM ethidium bromide) and visualized under UV transillumination (Gel Doc EZ imager, Biorad laboratories, USA) [18]. *Enterococcus faecium* ATCC 51559 and *Enterococcus faecalis* ATCC 51299 were used as *van*A and *van*B positive control, respectively. Meanwhile, *Enterococcus faecalis* ATCC 29212 was used as *van*A/B negative control strain.

2.4. 16S rRNA gene sequencing

All confirmed VRSA isolate underwent the amplification of partial (1.5 kb fragment) sequence of the 16srRNA gene. This process involved specific primers and reaction condition. The amplified products were then sequenced by 1st Base (www.Base.asia.com) using sanger sequencing techniques (Supplementary Table 1b). The resulting sequences were compared to the NCBI database to verify their identity.

2.5. Biocides susceptibility testing

To access biocide susceptibility, the Minimum inhibitory concentration (MICs) of benzalkonium chloride 20 % (BAC), 100 % potency of cetrimide (CET), and chlorhexidine gluconate (CHG) were determined using microdilution broth (Mueller Hinton broth (Merck, Germany)). The MICs value ranged from 0.25 μg/mL to 128 μg/mL as per guidelines of Clinical and Laboratory Standards Institute (CLSI). The bacterial inoculum was adjusted to a final concentration of 1×10^8 CFU/ml by spectrophotometric (ThermoFisher scientific, USA) measurement at 625 nm with observance between 0.8 and 1.3 [13]. *S. aureus* ATCC 6538 was used as a negative control and served as baseline for MICs. Any values above this threshold considered as tolerance concentration. Additionally, The MIC_{50} and MIC_{90} concentrations were also determined.

2.6. Detection of biocide resistant genes

All VRSA isolates were screened for the presence of BRGs (*cep*A, *qac*A, and *qac*E) by singleplex PCR using specific oligonucleotide primer sequences (Supplementary Table 1c). The *cep*A gene is associated with reduced susceptibility to CHG, whereas *qac*A and *qac*E genes are known for their decreased susceptibility to BAC and CET.

The genomic DNA from VRSA isolates was extracted using QIAamp DNA Mini kit (Qiagen, USA) as per manufacturer's instructions. Each reaction contained 12.5 μL master mix (AccuPrime, Invitrogen, USA), for each primer; 1 μL (10 pmol) of forward primer, 1 μL (10 pmol) of reverse primer, 2 μL of DNA extract and final volume was 25 μL by adding sterile H_2O. The thermocycler from Applied Biosystems (ThermoFisher, USA) was programmed for *qac*A, and *qac*E genes with initial denaturation step 96 °C for 3 min, followed by 25 cycles of 95 °C for 20 s, annealing step 53 °C for 20 s, extension step 72 °C for 20 s, a final extension step at 72 °C for 5 min. However, for *cep*A annealing temperature was set to 66 °C [13,19].

2.7. Efficacy testing of routinely used biocide

BAC, CET and CHG were selected as biocidal agents due to their frequent and widespread use in healthcare systems globally, including the studied hospital [20]. To access their efficacy against the isolated VRSA, a suspension test was used as per European Standard Guidelines [21]. Bacterial test suspensions were prepared by seeding sterile nutrient broth with an isolated colony and incubating under rotary conditions (125 rpm) for 12 h at 37 °C. Bacterial cell counts were adjusted to 1×10^{10} CFU/ml, using sterile saline solution. Chemical test solutions of BAC (20 %, Sigma USA), CET (100 %, Sigma USA) and CHG (20 %, Sigma USA) were prepared. Before testing, all reagents were equilibrated to the test temperature of 20 °C using the water bath. Subsequently, 8 mL test products (BAC, CET, CHG) were transferred separately to sterile containers with 1 mL sterile water. Afterward, 1 mL microbial suspension containing 1×10^{10} bacterial cells followed by 1 mL interfering substance (3.0 g/L bovine serum albumin) (Sigma USA) was

added to each container and incubated for 0–30 min while mixing at 20 °C. At set intervals of 5, 15, and 30 min, 1 mL test mixture was transferred into a tube containing 8 mL neutralizer (30 g/L polysorbate 80 + 3 g/L lecithine/l-a-phosphatidylcholine from egg yolk) (Sigma USA) and 1 mL sterile water. Samples were mixed and incubated in the water bath for 5 min. After neutralization, 100 μL bacterial suspension was transferred onto agar plates in triplicate and incubated at 37 °C for 24 h. Surviving cells of treated organisms were counted to determine the level of bacterial inactivation following exposure to the test solutions compared with the untreated control. For compliance with this test, test chemicals must achieve a 10^5 bacterial cell and 10^4 bacterial cell reduction in treatment time <5 min and <15 min, respectively [20]. The suspension of above mentioned neutralizer and sterile water without test products served as a negative control for the test.

2.8. Efflux pump inhibitor and reduced biocide susceptibility

To ensure the potential presence of efflux pumps, The MICs of BAC, CET and CHG were accessed in the presence of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (10 mg/L Sigma, USA) as a pump inhibitor [22]. This was undertaken to observe any potential reduction in the MICs of the biocides. The presence of efflux pumps would be confirmed by a decreased in the MICs value of the biocides in the presence.

2.9. Detection of efflux pump resistant genes

All VRSA isolates were screened for the presence of efflux pump resistant genes (EFPGs) (*nor*A, *sep*A, *mep*A and *mde*A) by singleplex PCR using specific oligonucleotide primer sequences (Supplementary Table 1d). The genomic DNA from VRSA isolates was extracted using QIAamp DNA Mini kit (Qiagen, USA) as per manufacturer's instructions.

Each reaction contained 12.5 μL master mix (AccuPrime, Invitrogen, USA), for each primer; 1 μL (10 pmol) of forward primer, 1 μL (10 pmol) of reverse primer, 2 μL of DNA extract and final volume was 25 μL by adding sterile H_2O. The thermocycler from Applied Biosystems (ThermoFisher, USA) was programmed for *nor*A gene was initially denatured at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 45–55 s and extension at 72 °C for 55 s to 1 min, followed by a step of final extension at 72 °C for 5 min. However, for *sep*A, *mep*A and *mde*A annealing temperature was set to 61 °C [23].

2.10. Statistical analysis

Variables normality was evaluated using Kolmogorov–Smirnov test. Comparisons between groups were assessed using the Kruskal–Wallis test followed by a post hoc Dunn's multiple comparison test due to the small data set. Biocides efficacy testing involved triple repetitions with three plate replicates per data point, resulting in a mean result for each experimental group. The log reduction was calculated as the log of the ratio of the concentration of the untreated and treated samples (log [nontreated/treated]) and represented as percentage loss in viability. Student t tests were performed, with statistical significance set as $p < 0.05$. Analysis employed, SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) for statistical computation. Antibiotic susceptibility pattern was analyzed through clustering, enhancing proximity, followed by a heat map display of permuted data. A hierarchical clustering heat map of antibiotic resistance profiles for isolates was constructed using XLSTAT (Addinsoft, New York, USA).

3. Results

Isolation, identification, and antibiotic susceptibility pattern of vancomycin resistant *S. aureus* isolated (VRSA) from frequently touched surfaces of ICU ward.

To determine the prevalence of VRSA isolates within the ICU ward. A total of twenty-three swab samples were obtained from three

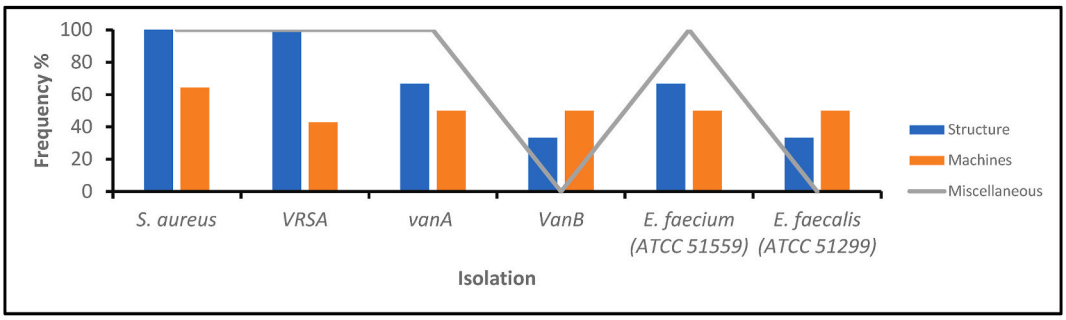

Fig. 2. Isolation and identification of VRSA. Samples from three frequently touched sites i.e., 1) Structure 2) Machines and 3) Miscellaneous subjected for *S. aureus* isolation on selective media, phenotypic detection for VRSA by antibiotic disc diffusion method as per CLSI and further PCR detection for *van*A or *van*B to confirmed VRSA. The results were showed in terms of percentage frequency (Isolates frequency/total number of isolates × 100).

primary (machine, structure, and miscellaneous) high contact sites of the studied ward. Subsequently, these samples were undergone for isolation as well as phenotypic and molecular identification. We found 72.8 % (n = 18/23) were confirmed as *S. aureus* and 83.3 % (n = 15/18) showed phenotypic resistance to vancomycin. According to PCR analysis 72.2 % (n = 13/18) of this *S. aureus* had the *van*A or *van*B gene and with no significant difference across three sites and internal control ($p > 0.05$) (Fig. 2). Notably, the frequency of isolation was significantly highest among the samples from machines in comparison to the other sites (structure and miscellaneous) ($p < 0.05$). These results suggest machines were the most contaminated site and potentially contributing to the transmission of hospital acquired infection (HAIs) linked with VRSA. Moreover, to determine the antibiotic susceptibility and similarity pattern between VRSA isolates. The disk diffusion method was used, and antibiotic resistance profile were analyzed through hierarchical clustering. The VRSA isolates exhibited complete resistance to several antibiotics, including oxacillin (100 %), cefoxitin (100 %), vancomycin (100 %), doxycycline (100 %), and clindamycin (100 μg). Conversely, azithromycin and linezolid (26 %) were the highest sensitive antibiotic against VRSA isolates (Fig. 3) (Supplementary Fig. 1). These results suggest VRSA isolates were resistant to multiple antibiotics. Hierarchical clustering within the heat map showed three primary clusters (A, B and C). Cluster A grouped five VRSA isolates from machines [(n = 2), USA44 and USA45] and miscellaneous sites [(n = 3), USA50, USA51 and USA53]. Notably, USA44 and USA45 exhibited the highest antibiotic susceptibility similarities within cluster A. While cluster B also included five VRSA isolates from structure [(n = 4), USA39, USA40, USA41, and USA42] and miscellaneous sites [(n = 1), USA52]. USA42 and USA52 exhibited the most pronounced antibiotic susceptibility similarity within cluster B. Furthermore, cluster C consists of four VRSA isolates from structure [(n = 1), USA43], machine [(n = 2), USA46 and USA47] and miscellaneous sites [(n = 1), USA49]. USA43 and USA47 displaying the highest similarity for antibiotic susceptibility pattern within cluster C. Conversely, USA48 did not show any significant linkage in hierarchical clustering (Fig. 3). Overall, these clustering patterns reveal probable sources and modes of spread within each cluster, pointing to distinct pathways and mechanisms of antibiotic resistance transmission across the VRSA isolates.

Distribution frequency of biocide resistant genes (BRGs) and biocides susceptibility values of vancomycin resistant *Staphylococcus aureus* (VRSA) isolates.

For effective control of VRSA dissemination within the ICU. We assessed biocide susceptibility and the distribution of BRGS (*cep*A, *qac*A, and *qac*E) through microdilution and singleplex PCR method. We hypothesized there is an association between BRGS and biocide susceptibility. We found that *cep*A and *cep*A in combination with *qac*A were the most predominant BRGs. Moreover, the isolates from structure site (n = 3) harboring highest BRGs frequency (100 %) with least (16.6 %) among isolates from miscellaneous site (n = 6) (Fig. 4). Overall, these results suggest structure site provide a favourable environment for the dissemination of BRGs within the ICU. In addition, Regarding the MICs values of VRSA isolates against three biocides (BAC, CET and CHG) ranged from 8 to 128 μg/mL. No

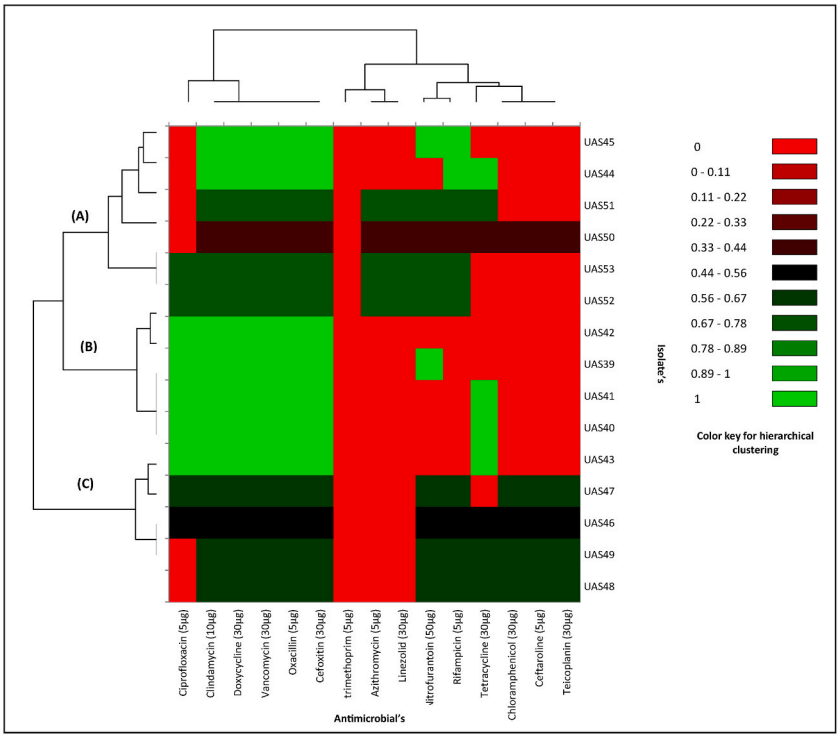

Fig. 3. Heatmap showing hierarchical clustering of isolates antibiotic resistance profiles.
Antibiotic susceptibility and similarity pattern between VRSA isolates were examined through single linkage clustering and heat map was generated using XLSTAT. Cluster analysis of AMR profiles, in the horizontal axis and in the vertical axis are the VRSA isolates obtained in this study. Overall, three main clusters were found.

reduced susceptibility to any of biocides was observed among the VRSA isolates from both machine and miscellaneous sites. However, the isolates from structure site [(n = 3), UAS41, UAS42 and UAS43] showed reduced susceptibility against BAC (8–32 μg/mL).These results suggest BAC was the most effective disinfectant against the VRSA isolates from structure site (**Table 1**). Furthermore, results of MIC50 and MIC90 obtained through the microdilution broth method confirmed that BAC was the most effective while CET exhibited the least effectiveness (Fig. 5). Moreover, there was no significant mean variance difference between presence or absence of BRGs and high MICs values of test isolates all three sites (Table 1). These results suggest the biocide susceptibility is not influenced by BRGs, but there could be potential association between BRGs and EFPGs.

3.1. *Efficacy testing of routinely practiced chemical disinfectants for ICU ward*

Further we assessed the potential efficacy of the studied disinfectant and to verify that BAC exhibited the highest effectiveness, the suspension method was used. We found that the VRSA test isolates of structure site showed maximum log 9 cell reduction against BAC as compared to CET and CHG. In terms of prominent level of cells death results indicates that BAC were the most effective disinfectant against the VRSA with significant difference to *S. aureus* ($P < 0.05$) and similar trend was observed among all three sites (structure, machines and miscellaneous) (Fig. 6). These results are helpful to overcome the selective pressure of biocide resistance and suggest appropriate use of disinfectant for the prevention of HAIs linked with VRSA.

Frequency of efflux pump genes mediating biocides resistance in vancomycin resistant *Staphylococcus aureus* (VRSA)

To determine the association between BRGs and EFPGs on biocide susceptibility of BAC, CET and CHG, we used CCCP efflux pump inhibitor. We found that CCCP efflux pump inhibitor had a direct significant effect on MICs of BAC, CET and CHG in the presence of BRGs and EFPGs. Further we found that VRSA isolates from structure site (UAS41, UAS42 and UAS43 showed maximum twofold MICs reduction with 10 mg/l CCCP efflux pump inhibitor in the presence of *nor*A + *sep*A (EFPGs) (Table 1). Furthermore, we observed that the isolates with *nor*A and *nor*A + *sep*A (EFPGs) confirming maximum BRGs (Table 2). These results suggest EFPGs had a significant association with BRGs.

4. Discussion

Microbial colonization on inanimate surfaces is responsible for the increased incidence of HAIs among patients admitted to ICU [24]. The reported prevalence of HAIs in ICU in developing countries is 2–20 time higher than those of developed countries [25]. We found high contamination frequently touched machines/instruments with *S. aureus* (Fig. 2). This frequency is higher than reported by other studies and illustrates how *S. aureus* contamination rates differ amongst hospitals [26]. This could be associated with complex hospital wards environment, patient occupancy, prolonged stay, and other infection control practices [27]. *S. aureus* is common human pathogens and significantly associated with HAIs [25] particularly VRSA in ICU (Fig. 2). This finding might be concerning given the rise in VRSA infections. Detection of VRSA is a matter of concern and requires special attention because vancomycin is the last therapeutic option for treating methicillin-resistant *Staphylococcus aureus* (MRSA) [28]. Various studies from region have reported the emergence of VRSA isolates in clinical samples [29]. VRSA contamination on ICU objects offers a significant risk of HAIs because patients admitted in ICU are often immunocompromised and involved in direct contact with frequently touched instruments [30]. Therefore, Identification of sites colonized by VRSA and understanding the reason of survival would minimize the transmission among patients by introducing effective disinfection and sanitation practices. Thus, helping in reducing incidence of VRSA linked HAIs and

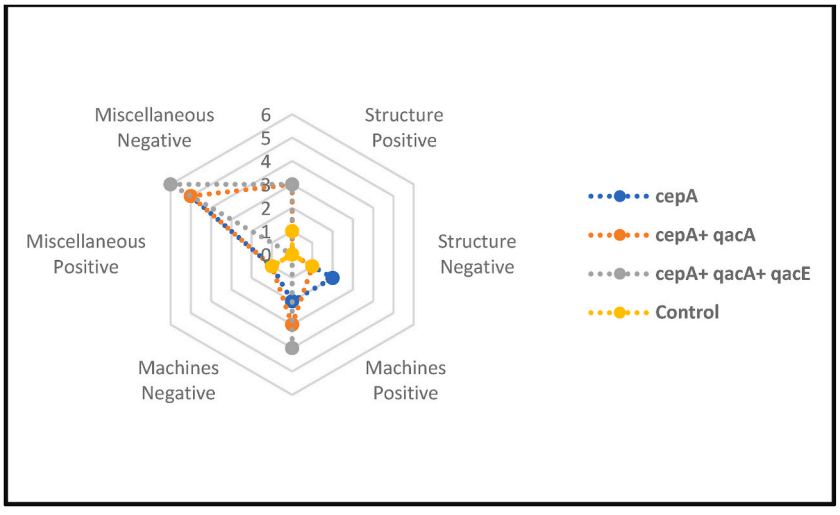

Fig. 4. Frequency distribution of biocide resistant genes (BRGs) among vancomycin resistant *staphylococcus aureus* (VRSA) isolates. Biocides resistance genes (*cep*A, *qac*A, and *qac*E) were targeted by using singleplex PCR. *cep*A found to be dominant BRGs. The results were displayed in terms of percentage frequency (Isolates frequency/total number of isolates × 100).

Table 1
Effect of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (10 mg/l) on minimum inhibitory concentration (MICs) of benzalkonium chloride (BAC), cetrimide (CET) and Chlorhexidine gluconate (CHG) in the presence of biocide resistant genes (BRGs) and association between efflux pump resistant genes (EFPGs). Key; B. machine (biometric machine), N. counter (nursing counter), and P. counter (pharmacy counter). In the column, the presence of the respective genes is displayed by blue color, while the absence of genes is indicated by orange color. Furthermore, the comparison of minimum inhibitory concentration (MICs) of benzalkonium chloride (BAC), cetrimide (CET) and chlorhexidine gluconate (CHG) and biocide resistant genes (BRGs) showed non-significant ^{NS} association. The result is significant at $p < 0.05$*.

Categories	Isolation sites	Isolates	VRSA		Biocide and efflux pump resistant genes							MIC µg/mL			Effect of CCCP (10 mg/l) on MIC		
			BRGs					EPRGs				Biocides			Biocides		
			vanA	*vanB*	*cepA* ^{NS}	*qacA* ^{NS}	*qacE* ^{NS}	*norA* *	*sepA* *	*mepA* *	*mdeA* *	BAC ^{NS}	CET ^{NS}	CHG ^{NS}	BAC *	CET *	CHG *
Structure	Paeds table	UAS41										8	16	16	2	4	2
	Floor	UAS42										8	32	32	2	4	2
	Switches	UAS43										32	64	128	4	8	4
Machines	Elevator	UAS44										8	16	32	4	4	4
	B. machine	UAS45										16	32	16	4	4	4
	Ventilator	UAS46										64	128	64	8	8	8
	Nebulizer	UAS47										16	32	128	8	8	8
	Chairs	UAS48										8	16	16	8	8	8
Miscellaneous	Lights	UAS49										8	32	32	4	8	8
	Handles	UAS50										16	64	128	8	8	8
	N. counter	UAS51										64	64	128	4	8	8
	P. counter	UAS52										32	16	32	4	8	8
	Paeds table	UAS53										8	32	64	4	8	16
E. faecium (ATCC 51559)												4	16	4	2	8	4
E. faecalis (ATCC 51299)												4	8	8	2	4	4

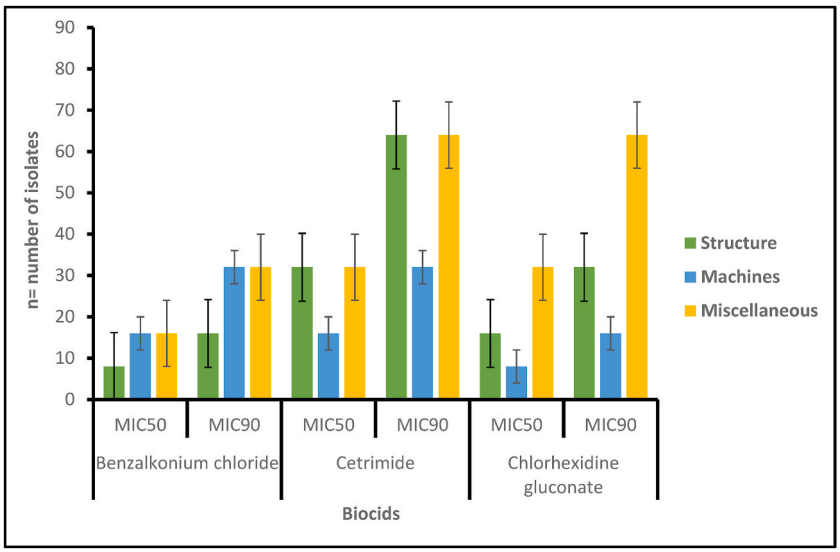

Fig. 5. MIC50 and MIC90 of (A) benzalkonium chloride 20 % (BAC) (b) 100 % potency of cetrimide (CET) and (c) chlorhexidine gluconate (CHG) solutions.

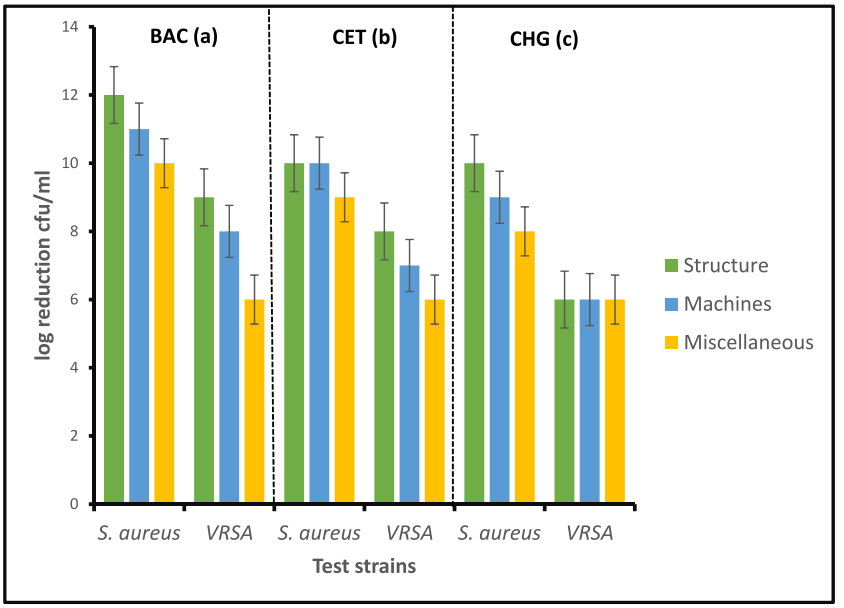

Fig. 6. Efficacy testing of routinely used disinfectant. Bactericidal suspension tests on VRSA test strains with (A) benzalkonium chloride 20 % (BAC) (b) 100 % potency of cetrimide (CET) and (c) chlorhexidine gluconate (CHG) solutions at a treatment time of 30 min (± standard deviation) among all isolation sites. Results show log reductions in viability (colony forming units) of test strains in the presence of 3.0 g/L bovine serum albumin (inhibitory substances) compared with *S. aureus*. A, B and C indicate significant difference at $P \leq 0.05$ among VRSA and *S. aureus*.

Table 2
Frequency distribution of efflux pump resistant genes (EFPGs) among vancomycin resistant *staphylococcus aureus* (VRSA) isolates.

Gene's	Isolation sites						Control			
	Structure (n = 3)		Machines (n = 4)		Miscellaneous (n = 6)		*E. faecium* (ATCC 51559) (n = 1)		*E. faecalis* (ATCC 51299) (n = 1)	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
norA	3	0	2	2	4	2	0	1	0	1
norA + *sepA*	3	0	2	2	4	2	0	1	0	1
norA + *sepA* + *mepA*	0	0	0	0	2	4	0	1	0	1
norA + *sepA* + *mepA* + *mdeA*	0	0	0	0	1	5	0	1	0	1

associated multiple antimicrobial resistance (AMR) in ICU. Our investigation revealed occurrence of AMR within VRSA isolates from the ICU environment (Fig. 3). The rise of AMR has emerged as a significant global health concern. The occupancy and mobility of patients plays a crucial role in the process of disseminating AMR. We collect these AMR carrying VRSA isolates while patients were present. These findings suggest potential sources of AMR dissemination could be from patients to hospital environment. However, to obtain precise understanding of AMR dissemination within studied ward, further studies based on genome analysis are necessary. Therefore, keeping hospitals clean and free from contamination has traditionally been considered a matter of aesthetics. Environmental surfaces in all hospitals are cleaned or disinfected regularly. However, the abuse and misuse of disinfectants provides selective pressure on bacterial cells, causing them to become resistant. This selective pressure is applied to both commensal and pathogenic bacteria, giving rise to resistant populations via vertical and horizontal gene transfer [31]. It has been known for a long time that bacteria can resist biocides, such as quaternary ammonium and chlorhexidine [32]. However, only a limited number of studies describe the distribution of BRGs, biocides susceptibility breakpoint and their efficacy data related to VRSA environmental strains. Specifically, there are no reports available relating to Pakistan and none of with respect to isolation site. For the effective BRGs dissemination control within a hospital setting it critical to find out the source. We found structure site as a source of BRGs with high prevalence of *cep*A. Almost in every hospital structure site is under massive selective pressure of disinfectant and experience direct patients and health workers contact [33]. This explains the high BRGs prevalence. In the current study, BRGs prevalence results are in line with the studies from different countries including United Kingdom, Saudi Arabia, Iraq, Iran, and China, where high prevalence of *cep*A gene has been reported [13,34,35] (Fig. 4). These studies, however, had investigated different bacteria with difference in sample size. According to the published reports, the *cep*A gene confers reduce susceptibility to chlorohexidine while *qac*A and *qac*E genes are known for their decreased susceptibility to quaternary ammonium compounds [36,37]. Similarly numerous different *qac* genes have been described among both environmental and clinical bacteria [38]. Despite the rising significance of VRSA as nosocomial pathogen

there are insufficient number of studies on *cep*A, *qac*A and *qac*E genes in environmental isolates. However, there are many studies available with respect to *Klebsiella*, *Pseudomonas*, *Acinetobacter,* and *Staphylococcus* species [11,13,39]. A study from Turkey on coagulase negative *S. aureus* reported high prevalence of *qac*A. Furthermore, there was no significant difference between presence or absence of BRGs and high MIC values of test isolates of all three sites and these results are in concise with Azadpour et al. and A'shimi et al. (Table 1) [40,41]**.** Therefore, the distribution and transmission of bacterial reduced susceptibility to biocides is likely to be affected and constrained by biological, physical, and socio-economic factors and these vary among different countries, regions, and communities. It's crucial to realise that a rise in the MIC values for biocides does not necessarily indicate "resistance," as these substances can be used at high concentrations without posing a hazard to health [42]. Therefore, for pathogens showing an increased MIC to a biocide, the terms "reduced susceptibility" or "increased tolerance" are more appropriate. In this study we found reduced susceptibility to BAC in concise with the results of MIC_{50} and MIC_{90} (Fig. 5) (Table 1).

Based on these results, we suggest that BAC was the most effective biocides against the VRSA and further confirmed by suspension test method (Fig. 6). These results are helpful to overcome the selective pressure of biocide resistance and suggest appropriate use of disinfectant for the prevention of HAIs. Resistance to biocides is less common and typically results from cellular changes that impact on biocide accumulation, including cell envelope changes that limit uptake, or expression of efflux mechanisms [43]. Further, we investigated the role efflux pump influencing the biocide resistance in VRSA. We found significant role of efflux pump in developing biocide resistance **(Table 1)**. Contrastingly, another study on *Klebsiella pneumoniae* demonstrate no effect on CCCP on MICs of tested biocide [11]. Furthermore, the association of EFPGs and BRGs was not only evaluated phenotypically but genotypic evidence has been provided by evaluating the related genes. Previous studies have documented the role of *nor*A EFPG as a determinant of biocide resistance genes (*qac*A and *qac*E) in clinical *S. aureus* isolates [44]. While in this study, we found *nor*A along with sepA (EFPGs) confirming maximum BRGs frequency in VRSA **(Table 2)**. None of the reports is available with respect to VRSA. This study exclusively focused on evaluating the effects of BRGs, EFPGs and biocides MIC against VRSA isolates. We do not intend to undertake genomic studies for comprehensive examination of spa typing and MLST of VRSA isolates and we recognize this as limitation of our study.

5. Conclusion

Overall, from this study we concluded that BAC was the most effective disinfectant against VRSA. *nor*A and *nor*A + *sep*A EFPGs had a significant association with BRGs. However, there was no significant variance difference observed between presence or absence of BRGs and biocide MICs. To our knowledge, this study is the first report showing distribution of BRGs and their biocide MIC values with efficacy testing and role of EFPGs towards BRGs among VRSA isolates from Pakistan. Consequently, there is a need for a large-scale study, using isolates from different hospitals, to confirm the trend of reduced susceptibility to biocides of other nosocomial pathogens. Proper use of basal biocides can be effective in preventing and controlling HAIs. A further experiment needs to be performed to determine the other genetic mechanisms that contribute to reduced biocide susceptibility in VRSA.

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Data availability

All analyzed data within this study can be obtained from the corresponding author on request.

Ethical approval

The Institutional Ethical Review Committee of the Faisalabad Institute of Cardiology (FIC) approved the study (letter no. 17–2019/DME/FIC/FSD).

Consent to participate

This study does not involve any patients.

CRediT authorship contribution statement

Muhammad Umer Asghar: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Noor Ul Ain:** Investigation, Writing - review & editing. **Arsalan Haseeb Zaidi:** Supervision. **Muhammad Tariq:** Supervision, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22120.

References

[1] M. Zarpellon, A. Gales, A. Sasaki, G. Selhorst, T. Menegucci, C. Cardoso, L. Garcia, M. Tognim, Survival of vancomycin-intermediate Staphylococcus aureus on hospital surfaces, J. Hosp. Infect. 90 (2015) 347–350.
[2] B.P. Howden, A.Y. Peleg, T.P. Stinear, The evolution of vancomycin intermediate Staphylococcus aureus (VISA) and heterogenous-VISA, Infect. Genet. Evol. 21 (2014) 575–582.
[3] W.H. Organization, The Selection and Use of Essential Medicines: Report of the WHO Expert Committee, 2017 (Including the 20th WHO Model List of Essential Medicines and the 6th Model List of Essential Medicines for Children), World Health Organization, 2017.
[4] Y. Wada, A.A. Irekeola, E.N.S. EAR, W. Yusof, L. Lih Huey, S. Ladan Muhammad, A. Harun, C.Y. Yean, A.R. Zaidah, Prevalence of vancomycin-resistant Enterococcus (VRE) in companion animals: the first meta-analysis and systematic review, Antibiotics 10 (2021) 138.
[5] Y. Cong, S. Yang, X. Rao, Vancomycin resistant Staphylococcus aureus infections: a review of case updating and clinical features, J. Adv. Res. 21 (2020) 169–176.
[6] K. Vickery, A. Deva, A. Jacombs, J. Allan, P. Valente, I.B. Gosbell, Presence of biofilm containing viable multiresistant organisms despite terminal cleaning on clinical surfaces in an intensive care unit, J. Hosp. Infect. 80 (2012) 52–55.
[7] D.J. Weber, W.A. Rutala, Understanding and preventing transmission of healthcare-associated pathogens due to the contaminated hospital environment, Infect. Control Hosp. Epidemiol. 34 (2013) 449–452.
[8] M. Irfan, A. Almotiri, Z.A. AlZeyadi, Antimicrobial resistance and its drivers—a review, Antibiotics 11 (2022) 1362.
[9] A. Amsalu, S.A. Sapula, M. De Barros Lopes, B.J. Hart, A.H. Nguyen, B. Drigo, J. Turnidge, L.E. Leong, H. Venter, Efflux pump-driven antibiotic and biocide cross-resistance in Pseudomonas aeruginosa isolated from different ecological niches: a case study in the development of multidrug resistance in environmental hotspots, Microorganisms 8 (2020) 1647.
[10] X. Song, L. Vossebein, A. Zille, Efficacy of disinfectant-impregnated wipes used for surface disinfection in hospitals: a review, Antimicrob. Resist. Infect. Control 8 (2019) 1–14.
[11] R. Goodarzi, R. Yousefimashouf, M. Taheri, F. Nouri, B. Asghari, Susceptibility to biocides and the prevalence of biocides resistance genes in clinical multidrug-resistant Pseudomonas aeruginosa isolates from Hamadan, Iran, Mol. Biol. Rep. 48 (2021) 5275–5281.
[12] D.J. Larsson, C.-F. Flach, Antibiotic resistance in the environment, Nat. Rev. Microbiol. 20 (2022) 257–269.
[13] R. Vijayakumar, T. Sandle, M.S. Al-Aboody, M.K. AlFonaisan, W. Alturaiki, S. Mickymaray, M. Premanathan, S.A. Alsagaby, Distribution of biocide resistant genes and biocides susceptibility in multidrug-resistant Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter baumannii—a first report from the Kingdom of Saudi Arabia, J. Infec. Public Health 11 (2018) 812–816.
[14] V. Kornelsen, A. Kumar, Update on multidrug resistance efflux pumps in Acinetobacter spp, Antimicrob. Agents Chemother. 65 (2021), e00514-e00521.
[15] C. Griffith, Surface sampling and the detection of contamination, in: Handbook of Hygiene Control in the Food Industry, Elsevier, 2016, pp. 673–696.
[16] M. Maharjan, A.K. Sah, S. Pyakurel, S. Thapa, S. Maharjan, N. Adhikari, K.R. Rijal, P. Ghimire, U. Thapa Shrestha, Molecular confirmation of vancomycin-resistant Staphylococcus aureus with vanA gene from a hospital in Kathmandu, Int. J. Microbiol. (2021) 2021.
[17] P. Wayne, CLSI Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing, M100-Ed31, February 2021 2021.
[18] S. Adkins, M. Burmeister, Visualization of DNA in agarose gels as migrating colored bands: applications for preparative gels and educational demonstrations, Anal. Biochem. 240 (1996) 17–23.
[19] N. Noguchi, M. Hase, M. Kitta, M. Sasatsu, K. Deguchi, M. Kono, Antiseptic susceptibility and distribution of antiseptic-resistance genes in methicillin-resistant Staphylococcus aureus, FEMS Microbiol. Lett. 172 (1999) 247–253.
[20] E. Meade, M. Garvey, Efficacy testing of novel chemical disinfectants on clinically relevant microbial pathogens, Am. J. Infect. Control 46 (2018) 44–49.
[21] T. Kasthuri, T.K. Swetha, J.P. Bhaskar, S.K. Pandian, Rapid-killing efficacy substantiates the antiseptic property of the synergistic combination of carvacrol and nerol against nosocomial pathogens, Arch. Microbiol. 204 (2022) 590.
[22] S.A. Baron, J.-M. Rolain, Efflux pump inhibitor CCCP to rescue colistin susceptibility in mcr-1 plasmid-mediated colistin-resistant strains and Gram-negative bacteria, J. Antimicrob. Chemother. 73 (2018) 1862–1871.
[23] S. Hassanzadeh, R. Mashhadi, M. Yousefi, E. Askari, M. Saniei, M.R. Pourmand, Frequency of efflux pump genes mediating ciprofloxacin and antiseptic resistance in methicillin-resistant Staphylococcus aureus isolates, Microb. Pathog. 111 (2017) 71–74.
[24] R.d.P. Menezes, L.d.A. Marques, F.F. Silva, N.B.S. Silva, P.G.V. Alves, M.A.d.S. Bessa, L.B.d. Araújo, M.P.A. Penatti, R.d.S. Pedroso, D.V. D.d. Brito Röder, Inanimate surfaces and air contamination with multidrug resistant species of Staphylococcus in the neonatal intensive care unit environment, Microorganisms 10 (2022) 567.
[25] D.R. Bhatta, S. Koirala, A. Baral, N.M. Amatya, S. Parajuli, R. Shrestha, D. Hamal, N. Nayak, S. Gokhale, Methicillin-resistant Staphylococcus aureus contamination of frequently touched objects in intensive care units: potential threat of nosocomial infections, Can. J. Infect Dis. Med. Microbiol. 2022 (2022).
[26] J.B. Yusuf, O.K. Okwong, A. Mohammed, K.S. Abubakar, A. Babayo, M.M. Barma, S. Ibrahim, A.I. Sulaiman, H. Hafiz, Z.S. Bello, Bacterial contamination of intensive care units at a tertiary hospital in Bauchi, Northeastern Nigeria, Am. J. Intern. Med. 5 (2017) 46.
[27] K.V. Sukhum, E.P. Newcomer, C. Cass, M.A. Wallace, C. Johnson, J. Fine, S. Sax, M.H. Barlet, C.-A.D. Burnham, G. Dantas, Antibiotic-resistant organisms establish reservoirs in new hospital built environments and are related to patient blood infection isolates, Commun. Med. 2 (2022) 62.
[28] W.-T. Liu, E.-Z. Chen, L. Yang, C. Peng, Q. Wang, Z. Xu, D.-Q. Chen, Emerging resistance mechanisms for 4 types of common anti-MRSA antibiotics in Staphylococcus aureus: a comprehensive review, Microb. Pathog. 156 (2021), 104915.
[29] M. Shekarabi, B. Hajikhani, A. Salimi Chirani, M. Fazeli, M. Goudarzi, Molecular characterization of vancomycin-resistant Staphylococcus aureus strains isolated from clinical samples: a three year study in Tehran, Iran, PLoS One 12 (2017), e0183607.
[30] G. Suleyman, G. Alangaden, A.C. Bardossy, The role of environmental contamination in the transmission of nosocomial pathogens and healthcare-associated infections, Curr. Infect. Dis. Rep. 20 (2018) 1–11.
[31] B. Cookson, H. Farrelly, P. Stapleton, R. Garvey, M. Price, Transferable resistance to triclosan in MRSA, Lancet 337 (1991) 1548–1549.
[32] R. Vijayakumar, T. Sandle, A review on biocide reduced susceptibility due to plasmid-borne antiseptic-resistant genes—special notes on pharmaceutical environmental isolates, J. Appl. Microbiol. 126 (2019) 1011–1022.
[33] E. Cloutman-Green, Infection Prevention and Control: Healthcare-Associated Infection, Hugo and Russell's Pharmaceutical Microbiology, 2023.
[34] A. Abuzaid, A. Hamouda, S. Amyes, Klebsiella pneumoniae susceptibility to biocides and its association with cepA, qacΔE and qacE efflux pump genes and antibiotic resistance, J. Hosp. Infect. 81 (2012) 87–91.
[35] Y. Chen, K. Liao, Y. Huang, P. Guo, H. Huang, Z. Wu, M. Liu, Determining the susceptibility of carbapenem resistant Klebsiella pneumoniae and Escherichia coli strains against common disinfectants at a tertiary hospital in China, BMC Infect. Dis. 20 (2020) 1–8.
[36] T. Wassenaar, D. Ussery, L. Nielsen, H. Ingmer, Review and phylogenetic analysis of qac genes that reduce susceptibility to quaternary ammonium compounds in Staphylococcus species, Eur. J. Microbiol. Immunol. 5 (2015) 44–61.
[37] M. Bischoff, J. Bauer, P. Preikschat, K. Schwaiger, G. Mölle, C. Hölzel, First detection of the antiseptic resistance gene qac A/B in Enterococcus faecalis, Microb. Drug Resist. 18 (2012) 7–12.

[38] M.R. Babaei, A. Sulong, R.A. Hamat, S.A. Nordin, V.K. Neela, Extremely high prevalence of antiseptic resistant Quaternary Ammonium Compound E gene among clinical isolates of multiple drug resistant Acinetobacter baumannii in Malaysia, Ann. Clin. Microbiol. Antimicrob. 14 (2015) 1–5.
[39] S. Ignak, Y. Nakipoglu, B. Gurler, Frequency of antiseptic resistance genes in clinical staphycocci and enterococci isolates in Turkey, Antimicrob. Resist. Infect. Control 6 (2017) 1–7.
[40] H. Mahmoudvand, M. Azadpour, J. Nowroozi, G. Goudarzi, Presence of qacEΔ1 and cepA genes and susceptibility to a hospital biocide in clinical isolates of Klebsiella pneumoniae in Iran, Trop. Biomed. 32 (2015) 109–115.
[41] M.H.N. A'shimi, A.G. Alattraqchi, F.M. Rani, N.I.A. Rahman, S. Ismail, F.H. Abdullah, N. Othman, D.W. Cleary, S.C. Clarke, C.C. Yeo, Biocide susceptibilities and biofilm-forming capacities of Acinetobacter baumannii clinical isolates from Malaysia, J. Infec. Dev. Countr. 13 (2019) 626–633.
[42] B. Meyer, B. Cookson, Does microbial resistance or adaptation to biocides create a hazard in infection prevention and control? J. Hosp. Infect. 76 (2010) 200–205.
[43] K. Poole, Mechanisms of bacterial biocide and antibiotic resistance, J. Appl. Microbiol. 92 (2002) 55S–64S.
[44] S. Hassanzadeh, M.R. Pourmand, R. Mashhadi, K. Ghazvini, Epidemiology of efflux pumps genes mediating resistance among Staphylococcus aureus; A systematic review, Microb. Pathog. 139 (2020), 103850.