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Study of the Resistance to Quaternary Ammonium-Based Disinfectants in *Escherichia coli* and *Staphylococcus aureus* Strains Carrying Third-Generation Cephalosporin Resistance

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Abstract

Multidrug-resistant bacteria pose a significant public health challenge, with the effectiveness of biocides in their eradication proving occasionally inadequate. This ineffectiveness highlights concerns over the potential correlation between biocide utilization and the rise in antibiotic resistance. Accordingly, this study is designed to examine the effectiveness of specific quaternary ammonium compound-based disinfectants against *Escherichia coli* and *Staphylococcus aureus* strains that are resistant to third-generation cephalosporins, particularly CTX-M type. The evaluation is based on performing antibiograms, followed by subjecting these strains to escalating concentrations of disinfectants with quaternary ammonium components. Additionally, the study involves screening for resistance genes to deepen our understanding of these resistance mechanisms. The outcomes revealed

that the strains under scrutiny were resistant to a multitude of antibiotics. The *Escherichia coli* strains in question also displayed markedly reduced susceptibility to the biocides being studied. Conversely, the *Staphylococcus aureus* strains were inhibited at notably low concentrations of biocides. Notably, the qacΔE1 gene was detected in two *Escherichia coli* strains. We have identified instances of cross-resistance between certain disinfectants and antibiotics. Specifically, certain E. coli strains demonstrated diminished susceptibility to the first biocidal agent. This resistance was supported by the presence of the qacΔE1 gene in these same strains.

Keywords: Escherichia coli, Staphylococcus aureus, multi-drug resistance, disinfectants, quaternary ammonium

Introduction

The rise of bacteria resistant to antibiotic therapies has become a critical issue for public health. Instances involving Methicillin-resistant Staphylococcus aureus (MRSA), Vancomycin-resistant Enterococci (VRE), strains of Mycobacterium tuberculosis with multi-drug resistance (MDR-TB), and various multidrug-resistant Gram-negative bacteria are increasingly reported, indicating a growing trend that concerns healthcare professionals globally [1].

The increasing use of antibacterial biocides in virtually all aspects of human activity must be considered within the broader context of the global exponential growth of pathogenic bacterial resistance to antibiotics, leading to the emergence of resistant bacteria in the environment [2, 3]. Consequently, bacterial resistance to various types of biocides has been reported in numerous scientific studies over the years [4, 5, 6, 7, 8].

Indeed, some researchers have posited a potential connection between the rise in antibiotic resistance and the use of biocides [9]. This phenomenon can be explained by common resistance mechanisms or through the co-selection of resistance genes (antibiotics-biocides) [10].

The aim of this work is to examine the effect of quaternary ammonium compound disinfectants on multidrug-resistant strains of E.coli and S.aureus, and to analyze the relationship between resistance to disinfectants and antibiotics by supplementing the study with a search for genes involved in disinfectant resistance.

Materials and Methods

Strains Studied: The study was conducted on multidrug-resistant *Escherichia coli* strains (4) and Methicillin-resistant *Staphylococcus aureus* strains (4), utilizing

reference strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

Biocides: The two biocides employed in this study belong to two representative families of disinfectants: Didecyldimethylammonium chloride (quaternary ammonium family) and Chlorhexidine digluconate (biguanide family). Biocide 1 (B1) and Biocide 2 (B2) contain ethanol as a component.

Measurement of the Minimum Inhibitory Concentration (MIC) of Strains to Biocides: In this study, the MIC for the two biocides was assessed using a micro-method with 96-well plates. Specifically, Biocide 1 was tested over a concentration range from 4% to 0.007%, with dilutions in half fractions. Similarly, Biocide 2 was tested over a concentration range from 50% to 0.09%.

The starting concentrations, 4% for the first biocide and 50% for the second, were determined by referring to the respective technical data sheets of the products. These values correspond to the thresholds from which each product is supposed to be effective, which justified our choice for determining their minimum inhibitory concentration (MIC) against the resistant strains studied.

Measurement of the Minimum Bactericidal Concentration (MBC): The MBC measurement aims to evaluate the bactericidal effect of a biocide, that is, to determine the minimum concentration of the product capable of killing bacteria, thus complementing the MIC assessment.

Using a sterile loop, a small amount of the contents from each well was sampled and streaked onto plates containing LB medium. The results were obtained after incubation for 24 hours at 37°C.

Antibiotics:

In total :

- 15 antibiotics were tested for E. coli: Amoxicillin (AML), Ticarcillin (TIC), Piperacillin/Tazobactam (TZP), Fosfomycin (FOT), Cefoxitin (FOX), Amoxicillin/Clavulanic Acid (AMC), Cefotaxime (CTX), Gentamicin (CN), Cefepime (FEP), Ertapenem (ETP), Kanamycin (K), Akamycin (AK), Ciprofloxacin (CIP), Sulfamethoxazole/Trimethoprim (SXT), and Ceftazidime (CAZ).

- And 16 antibiotics were tested for *S. aureus* : Penicillin (P), Oxacillin (OX), Tobramycin (TOB), Gentamicin (GEN), Erythromycin (ERY), Tetracycline (TC), Tigecycline (TGC), Linezolid (LZD), Fusidic Acid (FUS), Vancomycin (VA), Teicoplanin (TEC), Sulfamethoxazole/Trimethoprim (TSU), Fosfomycin (FOS), Clindamycin (CLI), Rifamycin (RIF), Levofloxacin (LVX).

Antibiogram : The antibiogram was performed using the disc diffusion method, employing a bacterial suspension adjusted to 0.5 McFarland standard.
Pre-impregnated discs with a specified amount of antibiotic were placed onto the surface of agar plates. The antibiotic diffuses from the disc into the agar, creating a concentration gradient. The diameter of the inhibition zone following incubation is measured to estimate the minimum inhibitory concentration. The susceptibility or resistance characteristics of the bacterial strain are then inferred from this analysis by comparing the diameters with predefined interpretive charts.
Genotypic Method : Bacterial DNA from E. coli strains was extracted using the thermal shock method. The detection of the qacE gene, a member of the qac gene family, was conducted using PCR on their DNA. The sequences of this gene were determined from broad-host-range plasmids that replicate within Gram-negative bacteria. PCR was performed in a final volume of 50µL. The separation of the various DNA fragments was carried out by agarose gel electrophoresis.

Findings:

Antibiotic Sensitivity Profiles

The sensitivity profiles to the tested antibiotics for the *E. coli* and *S. aureus* strains are presented respectively in Tables 1 and 2.

Table 1: Antibiotic Susceptibility Testing Results for *E. coli* Strains. R: Resistant, I: Intermediate.

	AML	TIC	FZP	FOT	FOX	AMC	CTX	CN	FEP	ETP	K	AK	CIP	SXT	CAZ
E.Coli 1	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R
E.Coli 2	I	R	R	R	R	R	I	I	R	R	R	I	R	R	R
E.Coli 3	R	R	R	R	R	R	R	R	R	R	R	I	R	R	R
E.Coli 4	R	R	R	R	I	I	I	R	R	R	R	R	R	R	R

The provided antibiogram table for four strains of *E. coli* shows that all strains exhibit complete resistance to a range of antibiotics including ticarcillin, piperacillin/tazobactam, fosfomycin, gentamicin, cefepime, ertapenem, kanamycin, ciprofloxacin, trimethoprim/sulfamethoxazole, and ceftazidime. There is a mixed response to amoxicillin, amoxicillin/clavulanic acid, cefoxitin, and cefotaxime, with some strains showing intermediate resistance.

Table 2: result of the antibiogram of S.aureus strains. R: Resistant, I: Intermediate, S: sensitive;

	P	OX	TOB	GEN	ERY	TC	TGC	LZD	FUS	VA	TEC	TSU	FOS	CLI	RIF	LVX
S.aureus 1	R	R	R	S	R	R	R	S	R	S	R	S	R	R	I	S
S.aureus 2	R	R	R	R	R	R	R	S	R	S	R	S	R	S	I	R
S.aureus 3	R	R	R	R	S	R	S	S	R	S	S	R	R	S	I	S
S.aureus 4	R	R	R	R	R	R	R	S	R	S	R	S	R	S	I	R

The antibiogram reveals that the Staphylococcus aureus strains tested exhibit a high level of resistance to several common antibiotics, including Penicillin, Oxacillin, Tobramycin, Erythromycin, Tetracycline, and Fosfomycin. However, they remain consistently sensitive to Gentamicin, Linezolid, Vancomycin, and Teicoplanin. The response to Tigecycline, Sulfamethoxazole/Trimethoprim, Fusidic acid, Rifampicin, and Levofloxacin is mixed, showing some degree of intermediate sensitivity or resistance.

Results of MIC and MBC

The aim of the study was to test the multi-drug resistant strains of E.coli and S.aureus for resistance to biocides. We exposed these strains to different concentrations of biocides. Table 3 shows the results of the MIC for the two biocides tested.

Table 3: MIC results of the studied strains against the tested biocides

	MIC of *Escherichia coli* in %		MIC of *Staphylococcus aureus* in %	
	B1	B2	B1	B2
Reference Strains	0.015	0.09	0.015	0.3
Strain 1	0.031	0.3	0.015	0.1
Strain 2	0.031	0.3	0.015	0.3
Strain 3	0.068	0.7	0.015	0.1
Strain 4	0.068	6.2	0.015	0.1

For Biocide 1, MICs for *E. coli* ranged from 0.015% to 0.068%, while *S. aureus* strains uniformly exhibited an MIC of 0.015%. Regarding Biocide 2, *E. coli* MICs varied between 0.09% and 0.7%, with a significant outlier at 6.2% for one strain. In contrast, all strains were inhibited at concentrations of 0.3% or less.

Biocidal action was bactericidal for some strains (where MIC equals MBC) and merely inhibitory for others, requiring higher concentrations for bactericidal effects. S. aureus strains were generally more susceptible to both biocides, with growth inhibition at low concentrations comparable to the reference strain *S. aureus* ATCC 25923, except for one strain which showed a marginally higher growth at 0.7%. Conversely, *E. coli* strains displayed reduced sensitivity to Biocide 1, with MBCs exceeding those of the reference. Moreover, Biocide 2 was less effective, with some *E. coli* strains capable of growth at concentrations as high as 6.2%.

Table 4: Minimum Bactericidal Concentration (MBC) results for the studied strains against the tested biocides

	MBC of Escherichia coli in %		MBC of Staphylococcus aureus in %	
	B1	B2	B1	B2
Reference Strains	0.015	0.3	0.068	0.3
Strain 1	0.068	1.5	0.015	0.1
Strain 2	0.068	0.7	0.015	0.7
Strain 3	0.068	0.7	0.015	0.3
Strain 4	0.068	6.2	0.015	0.1

Discussion

Our findings are in agreement with those reported by Chaidez et al. (2007), indicating that biocides containing quaternary ammonium compounds are effective against *Staphylococcus aureus,* while *Escherichia coli* showed susceptibility [11]. Similarly, our results align with Gottenbos's (2001) findings, which demonstrated that reference strains of *Staphylococcus aureus, Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* were sensitive to quaternary ammonium compounds [12].

In contrast, our study presents divergent results from those reported by Montagna et al (2019), which showed that disinfectants based on didécyl dimethyl ammonium chloride were ineffective against strains of *Staphylococcus aureus* [13].

The disparity in the results obtained could be ascribed to the antibiotic resistance exhibited by the strains under investigation. Russell's study in 2000 elucidates that hospital settings frequently deploy quaternary ammonium-based disinfectants such as benzalkonium chloride, cetylpyridinium chloride, cetrimide, and detizor for sanitation purposes and to curtail pathogen dissemination. This suggests that the pervasive application of quaternary ammonium compounds may induce a selective pressure conducive to the emergence of microorganisms resistant to such disinfectants [14].

Furthermore, Russell's insights from 1991 indicate that the variability in the susceptibility of microorganisms to disinfectants can be partly explained by the compositional differences in the bacterial envelope between Gram-positive and Gram-negative bacteria and even within the same bacterial species [15].

Expanding on this, Joly in 1995 posits that the primary target of most antiseptics and disinfectants is the cytoplasmic membrane, implying that inherent resistance is

likely tied to the level of protective integrity afforded by the membrane. The structural defenses such as an outer membrane in Gram-negative bacteria, a waxy coating in mycobacteria, or protective spore coats substantially bolster the resistance of microbial cells to disinfectant action [16].

Research discussing the combined resistance to quaternary ammonium compounds (QACs) and antibiotics often employs terms like cross-resistance or co-resistance, because it's conceivable that a single multidrug efflux pump could expel both QACs and antibiotics. Alternatively, alterations in the cell membrane structure might concurrently affect both types of agents. Moreover, genes encoding efflux pumps for QACs may be co-located with those conferring antibiotic resistance, whether through the production of an inactivating enzyme or an alteration of the intracellular target. Nonetheless, it is critical to underscore that literature pertaining to cross-resistance and co-resistance commonly describes pathogens with a combined tolerance to QACs and resistance to antibiotics.

Reflecting on the findings from our study, the different strains examined exhibited resistance to third-generation cephalosporins, such as CTX-M types, and a broader antibiotic resistance, suggesting a cross-resistance profile for *E. coli* strains that also demonstrated markedly reduced susceptibility to the biocides tested. This outcome aligns with Buffet-Bataillon et al.'s 2016 study, which described community and clinical isolates with a tolerance to QACs (diminished sensitivity) coupled with clinically defined antibiotic resistance. However, such cross-resistance was not observed in the *S. aureus* strains [17].

Investigation of the qacΔE1 gene:

Following the promising results from the minimum bactericidal concentration (MBC) method, which indicated a tolerance to quaternary ammonium compounds (QACs) among *E. coli* strains, a genotypic assay was conducted to search for the resistance gene associated with this phenotype, specifically the qacΔE1 gene.

The qacΔE gene is a member of the qac gene family. It encodes a protein consisting of 115 amino acids that was initially identified in *E. coli*. The targeted qacΔE1 gene is typically carried by integrons or plasmids that code for transmembrane proteins involved in the efflux pump system. Ortega et al. (2013) suggest that cross-resistance may arise from the expression of a single efflux pump that can actively expel both quaternary ammonium compounds and antibiotics [18].

The interrelationship between multiresistance in Gram-negative bacteria and the qac gene family, which confers resistance to quaternary ammonium compounds and chlorhexidine, is well-documented [19]. In the *E. coli* strains scrutinized in our study, the qacΔE1 gene was detected exclusively in strains 1 and 2. These particular strains demonstrated a pronounced resistance to quaternary ammonium-based

agents. This observation negates the potential for an intrinsic resistance and underscores the imperative to investigate additional acquired resistance mechanisms.

Conclusion

Addressing the rise and spread of antibiotic resistance is a major public health imperative. Recent discourse has heightened awareness around the potential connections between the use of biocidal agents and the development of antibiotic resistance, a relationship that could be instrumental in perpetuating resistance phenomena.

The premise of our research was to evaluate the resistance profile of multidrug-resistant strains of E. coli and S. aureus against biocides formulated with quaternary ammonium compounds. Through empirical testing, we have identified instances of cross-resistance between certain disinfectants and antibiotics. Specifically, certain E. coli strains demonstrated diminished susceptibility to the first biocidal agent, as evidenced by minimum bactericidal concentrations (MBC) exceeding those of the reference strain. Moreover, the second biocidal agent appeared to be ineffectual against these strains, which proliferated at concentrations as high as 6.2%.

Furthermore, the quaternary ammonium compound tested in our study was effective against *S.aureus* strains, which were inhibited at very low concentrations, not exceeding those of the reference strain *S. aureus* ATCC 25923.

Based on these findings, it can be deduced that the biocides in question are more potent against Gram-positive bacteria than Gram-negative ones.

Moreover, the detected resistance of *E. coli* strains to the quaternary ammonium compound prompted an investigation into associated resistance genes, notably the qacΔE1 gene. This gene was identified in two *E. coli* strains, with the cross-resistance observed underscoring the occurrence of plasmids harboring both beta-lactam resistance genes and quaternary ammonium resistance genes [19].

This resistance emphasizes the need for ongoing research into additional potential resistance genes and understanding their impact on the efficacy of disinfectant products. Such knowledge paves the way for the development of more effective preventive and therapeutic strategies.

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