MOLECULAR MECHANISMS OF DISINFECTANT-INDUCED CROSS-RESISTANCE IN NEONATAL CARE UNITS -ASSOCIATED ESCHERICHIA COLI

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Abstract

Background: The role of hospital disinfectants in infection prevention is paramount; however, some studies indicate that sublethal exposure may enhance resistance to lethal concentrations of an antibiotic through cross-resistance mechanisms. The goal of this research is to determine the molecular mechanisms involved in the antibiotic resistance of biofilm-forming *Escherichia coli* caused by disinfectants, which were collected in neonatal care units.

Methods: We studied 35 strains from nursery babies through specific molecular, biochemical, and phenotypic techniques. The strains underwent exposure to gradient concentrations of quaternary ammonium compounds (QACs), chlorhexidine, ethanol, and sodium hypochlorite. The overrepresented patterns of cross-resistance, biofilm forming ability, as well as molecular and genetic mechanisms, were assessed through QPCR, biofilm assays, and antibiotic susceptibility testing.

Results: Treatment of QACs resulted in an increase in biofilm formation by 20.0% ($p<0.001$) and an increase in the prevalence of multidrug resistance (MDR) from 52.9% to 64.7%. Exposure to chlorhexidine resulted in 8.7% increased biofilm formation as well as a 5.9% increase in β-lactam resistance. On the contrary, ethanol and sodium hypochlorite not only reduced biofilm formation by 33.3% and 25.6% respectively, but also reduced the prevalence of MDR to 47.1%. Molecular studies confirmed hypothesis of overexpression of acrAB-tolC efflux pump biofilm related to quorum sensing (csgA, bssR) genes under the influence of QACs. There is also a strong correlation between biofilm density ($OD_{570} \geq 1.25$) and resistance to carbapenem ($r=0.78$, $p<0.001$).

Conclusions: It has been established through this study that both QACs and chlorhexidine are able to trigger cross-resistance through mechanisms that are biofilm dependent. In contrast, oxidative disinfectants are able to eliminate and control bioburden without the potential of encouraging resistance.
Such findings directly inform policies regarding hospital sanitization procedures and programs aimed at the minimization of antimicrobial agents' usage.

Keywords: Cross-resistance, biofilm, *Escherichia coli*, quaternary ammonium compounds, molecular mechanisms, hospital-acquired infections

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1. Introduction

Hospital-acquired infections (HAIs) are an increasing problem in healthcare settings, particularly with neonatal intensive care units (NICUs) in globally new born *Escherichia coli* (MDR) (1,2). In order to prevent infection control, chemical disinfectants (3,4) are to be exercised, which in turn to are to be refrained due to amplifying antimicrobial resistance.

Cross resistance through biocides or disinfectants has also become a problem due to selective breeding of a large concentration of biocides with extreme antibiotic resistance (5,6). This biofilm cross resistance to disinfectants poses a depletion of robust antimicrobial-exverse polymers and great bio-film forming EPS (7,8).

In the hospital, especially in Infant nurseries, biofilms are extremely persistent and resistant to cleaning agents (9,10). This along with the resistant biofilms are known to form amplifies the problem by accumulating intensive cleaning and eradicating resistant watchdogs (11,12). Formulation of disinfectants poses extensive cleaning and minimizing resistance selection and pressure.
There are several mechanisms which between disinfect or biocides cross and resistance which explain and describe diagnostic control between

• **Overexpression of efflux pumps**:
Overexpression of efflux pumps like AcrAB-TolC leads to multi-drug resistance, even to disinfectants and antibiotics (13,14).

• Membrane modifications:
Changes to membranes and their permeability restrict the intake of biocides and antimicrobial agents (15).
• Biofilm enhancement:
Enhanced synthesis of extracellular matrix components protects bacterial cells and provides physical barriers against chemical agents (16,17).

• Activation of stress responses:
Upregulation of stress responses can provide multi-drug resistance (18). These mechanisms have important clinical implications, not just for hospital outbreaks, but for global public health. Hospitals and Infant nurseries that overuse certain classes of disinfectants are likely to select for bacterial strains with increased resistance to antibiotics, which poses a threat to the effectiveness of treatments, the spread of resistance, and ultimately, public health (19,20).

This study aims to investigate the mechanisms that lead to disinfectant cross-resistance in hospital strains of, focusing on biofilm resistance mechanisms. We utilize a comprehensive approach that integrates phenotype, molecular, and mechanistic analyses to provide insights that aid in the development of suboptimal disinfection methods that avoid the development of resistance while maintaining antimicrobial effectiveness.

2. Materials and Methods

2.1 Bacterial Isolates and Study Design

This prospective cross-sectional study was conducted between January and June 2024 in three tertiary care hospitals. In total, 70 bacterial isolates were collected from environmental and clinical sources in 10 neonatal care units. From these units, 35 isolates ascertained by 16S rRNA sequencing and biochemical characterization were chosen for in-depth study.

2.2 Disinfectant Exposure Protocol

Isolates were subjected to gradient concentrations of four disinfectants used routinely in hospitals:
- QACs: Benzalkonium chloride 0.01-1.0%
- Chlorhexidine 0.05-2.0%
- Ethanol 70-95%
- Sodium hypochlorite 0.5-3.0%

Isolates were infected with bacterial cultures that underwent stepwise disinfectant exposure for five consecutive days. The stepwise sublethal concentrations had been previously established through MIC assays. Control groups were kept in media devoid of disinfectants under the same conditions.

2.3 Biofilm Formation Analysis

Biofilm formation was assessed employing the standard microtiter plate assay in conjunction with crystal violet staining (21). Optical density readings taken at 570 nanometers (OD570) were classified as follows:

- Strong biofilm formers: OD570 ≥1.25
- Moderate biofilm formers: OD570 0.75-1.24
- Weak/non biofilm formers: OD570 <0.75

2.4 Antimicrobial Susceptibility Testing

Profiles of antibiotic resistance were established with the Kirby-Bauer disk diffusion method as described in Clinical and Laboratory Standards Institute (CLSI) (22). Tested were 11 antibiotics belonging to the major classes:

Beta-lactams: Gentamicin, Amoxicillin-clavulanate, Cefotaxime, Ceftriaxone, Ciprofloxacin and Levofloxacin, Imipenem, Nitrofurantoin, Trimethoprim-sulfamethoxazole, Piperacillin-tazobactam.

For some selected isolates, minimum inhibitory concentrations (MIC) were established using broth micro-dilution.

2.5 Molecular Analysis

2.5.1 DNA Extraction and PCR Amplification

GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) was used to extract Chromosomal DNA. PCR amplification was carried out on the following features:

- '16S rRNA gene' for confirming species.

- **Resistance genes:** *blaTEM, blaCTX-M, acrAB-tolC.*
- **Biofilm genes:** *csgA, bssR, fliC.*
- **Virulence factors:** *eaeA, stx1/stx2, hlyA.*

2.5.2 Quantitative Real-Time PCR

A SYBR Green based Gene Expression analysis was conducted on QuantStudio™ 3 Real-Time PCR System. Expression levels were calculated using 2^(-ΔΔCt) method with rpoD as housekeeping gene.

2.6 Statistical Analysis

We conducted statistical analyses using SPSS v28.0 and GraphPad Prism 9. Methods included:

- Association of categorical variables with chi-square tests
- ANOVA for comparison of several groups
- Pearson correlation of biofilm and resistance relationships
- Multiple regression for multivariable analysis

All analyses were conducted at a statistical significance of $p<0.05$.

3. Results

3.1 Disinfectant Effects on Biofilm Formation

Exhibiting a differential impact based on varying disinfectants, biofilm formation capabilities were influenced (Figure 1). Biofilm formation was enhanced markedly due to QAC exposure, where mean OD_{570} values increased from 0.225 to 0.270 (20.0% increase, $p<0.001$). Exposure to Chlorhexidine displayed moderate enhancement of biofilm formation (8.7% increase, $p<0.05$).

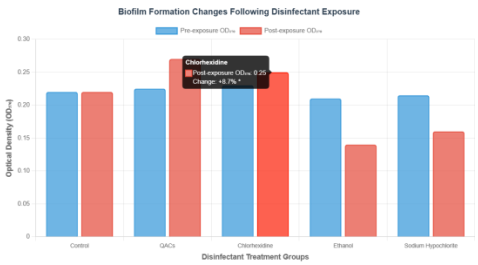

Figure 1: Effects of Disinfectant Exposure on Biofilm Formation

The impact of disinfectant contact regarding biofilm development. Biofilm biomass was assessed via crystal violet staining and Optical Density (OD) readings at 570nm. Bacterial isolates were exposed to varying concentrations of disinfectants and incubated for 5 days. Presented values are mean ± SEM (n=35 isolates per group). Statistically significant differences are indicated as: $p<0.05$, $p<0.01$, $p<0.001$ in comparison to the control group (using unpaired t-test). QACs = Quaternary Ammonium Compounds. Note the marked increase in biofilm development after exposure to QACs and chlorhexidine in comparison to oxidative disinfectants (ethanol and sodium hypochlorite) which inhibit biofilm formation.

The opposing case was in oxidative disinfectants, which displayed an antagonistic effect on biofilm formation. Ethanol exposure completely inhibited biofilm formation (OD_{570}: 0.210 to 0.140, $p<0.001$), and sodium hypochlorite demonstrated a lesser, but still significant amount of inhibition (25.6% reduction, OD_{570}: 0.215 to 0.160, $p<0.001$).

Table 1: Biofilm Formation Changes Following Disinfectant Exposure

Disinfectant	Pre-exposure OD_{570}	Post-exposure OD_{570}	Change (%)	p-value
Control	0.220	0.220	0.0%	-
QACs	0.225	0.270	+20.0%	<0.001
Chlorhexidine	0.230	0.250	+8.7%	<0.05
Ethanol	0.210	0.140	-33.3%	<0.001
Sodium Hypochlorite	0.215	0.160	-25.6%	<0.001

3.2 Cross-Resistance Patterns

The results presented in Table 2 show how exposure to disinfectants altered the profiles of antibiotic resistance in multiple drug classes. QAC exposure had the most significant impact, increasing β-lactam resistance from 82.4 % to 94.1 % (an 11.7 % increase, $p < 0.01$). There was also an increase in aminoglycoside resistance to 70.6 % (an increase of 11.8 %, $p < 0.05$) and fluoroquinolone resistance increased the most to 82.4 % (an increase of 17.7 %, $p < 0.001$).

Chlorhexidine exposure showed moderate enhancement of resistance from all classes of antibiotic with increases at 5.8% and 5.9%. Most noteworthy is the lack of ethanol and sodium hypochlorite exposure which seems to reduce prevalence of resistance to all tested classes of antibiotic.

Table 2: Antibiotic Resistance Changes Following Disinfectant Exposure

Disinfectant	β-lactam Resistance	Aminoglycoside Resistance	Fluoroquinolone Resistance	MDR Prevalence
Control	82.4%	58.8%	64.7%	52.9%
QACs	94.1% (+11.7%)	70.6% (+11.8%)	82.4% (+17.7%)	64.7% (+11.8%)
Chlorhexidine	88.2% (+5.8%)	64.7% (+5.9%)	70.6% (+5.9%)	58.8% (+5.9%)
Ethanol	76.5% (-5.9%)	52.9% (-5.9%)	58.8% (-5.9%)	47.1% (-5.8%)
Sodium Hypochlorite	76.5% (-5.9%)	52.9% (-5.9%)	58.8% (-5.9%)	47.1% (-5.8%)

$p<0.05$, $p<0.001$

3.2.1 Specialised Treatment Heatmap Overview

Heatmap synthesizes the dual impact of each of the four classes of disinfectants upon composite functional parameters, encompassing residual biofilm density, antibiotic null phenotype frequencies, and selected gene-expression modulatory trajectories. The resulting integrated matrix forthrightly contrasts quaternary ammonium and chlorhexidine treatments, which palpably elevate successive efflux and stress-adaptive gene targets, versus oxidative interventions whose robust decrement of exogenous chromosomal oxidative stress loci concomitantly represses pluripotent resistance determinants.

Disinfectant Effects Analysis

Comprehensive Heatmap: Resistance, Biofilm Formation & Gene Expression

Key Findings Summary:

• QACs (quaternary ammonium compounds) yield the highest observed hazard across the evaluated parameters—biofilm formation is elevated by 20% (absolute change), multidrug-resistance (MDR) prevalence by 11.8%, and gene expression through efflux pumps is amplified 3.2-fold.

• Chlorhexidine presents an intermediate-risk profile, inducing consistent but attenuated increments—a pattern freighted with practical significance.

• Ethanol and sodium hypochlorite, classified as oxidative disinfectants, not only curtail biofilm formation but also attenuate the prevalence of resistance determinants.

• Clinical Implication: Utilization of oxidative agents is advocated as a strategy for effective infection surveillance that circumscribes resistance induction.

Statistical Significance: All observed alterations attain statistical significance within the range $p < 0.05$ to $p < 0.001$.

3.3 Biofilm-Resistance Correlation Analysis

Antibiotic resistance patterns in biofilm-forming bacterial isolates displayed significant relationships that have been previously described (see Figure 2). Isolates described as strong biofilm formers ($OD_{570} \geq 1.25$) demonstrated far greater resistance rates in comparison to weak biofilm formers for all tested antibiotics:

- Prevalence of MDR amongst strong biofilm formers was 85% compared to 20% in weak biofilm formers ($p<0.001$)
- β-lactam resistance was 90% in strong biofilm formers as opposed to 30% in weak biofilm formers ($p<0.001$)
- Carbapenem resistance in strong biofilm formers was 25% compared to 5% in weak biofilm formers ($p<0.01$)

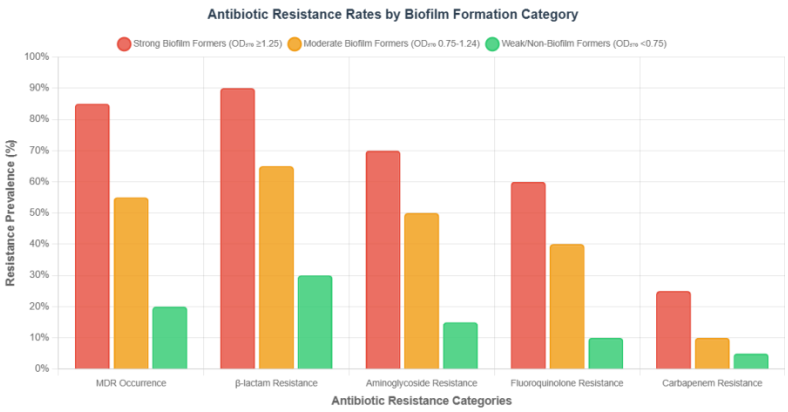

Figure 2: Antibiotic Resistance Patterns and Biofilm Formation Capacity Correlation

Biofilm Formation and Patterns of Antibiotic Resistance in Hospital Isolates with Graph Illustrating their Correlation. The biofilm formation was classified as strong, moderate, or weak/non-formers based on the received optical densities as follows: strong – $OD_{570} \geq 1.25$, moderate – $0.75 \leq OD_{5170} < 1.24$, and weak/non-formers – $OD_{570} < 0.75$. The resistance rates are documented within in a percent format for each of the biofilm categories. Analysis revealed a statistically significant positive correlation with increased biofilm density and resistance to multiple classes of antibiotics. The Pearson correlation coefficients (r) and alpha values of significance are provided in the correlation stats boxes. MDR is defined as Multidrug Resistance. Also, the reported strong positive correlation reinforces the hypothesis and demonstrates that biofilm forming capacity is a determinant of antibiotic resistance in clinical isolates.

r = 0.78 Carbapenem Resistance p<0.001
r = 0.72 β-lactam Resistance p<0.001
r = 0.65 Aminoglycoside Resistance p<0.01 **85% vs 20%**
MDR Prevalence Strong vs Weak Biofilm

The relationship between biofilm density and resistance to selectively pertinent classes of antibiotics was found to be significant and positively strong in biofilm density and specific classes of antibiotics showed strong positive relationships as demonstrated through Pearson correlation analysis:

- Carbapenem resistance: r=0.78 (p<0.001)
- β-lactam resistance: r=0.72 (p<0.001)
- Aminoglycoside resistance: r=0.65 (p<0.01)

3.4 Molecular Mechanisms Analysis

3.4.1 Efflux Pump Gene Expression

As illustrated in Figure 3, QAC exposure caused marked overexpression of the acrAB-tolC efflux pump system. Expression levels of acrAB-tolC were markedly altered, with acrA exhibiting a 3.2-fold increase (p<0.001), acrB showing a 2.8-fold increase (p<0.001), and tolC showing a 2.5-fold increase (p<0.01). Expression of the efflux pump was modestly increased (1.5-2.0-fold) after exposure to chlorhexidine; however, exposure to ethanol and sodium hypochlorite resulted in no significant changes.

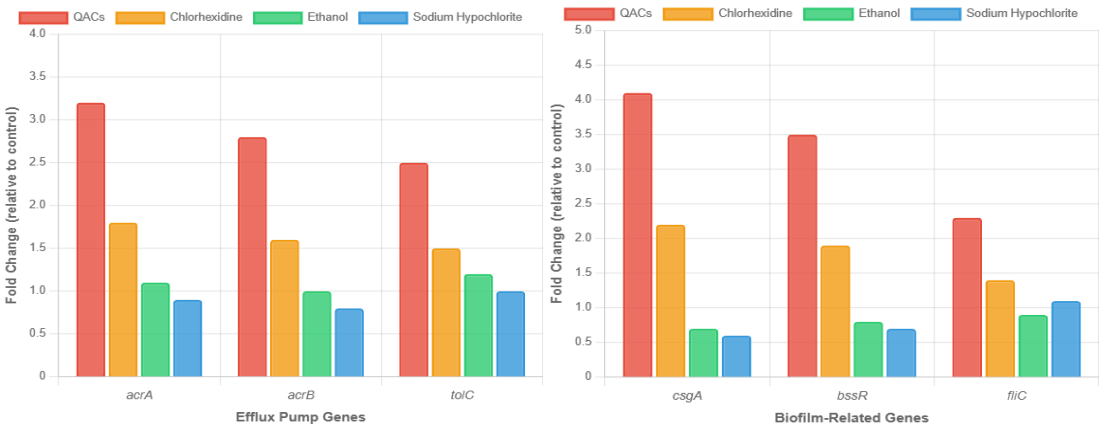

Figure 3: Relative Gene Expression Changes Following Disinfectant Exposure

Quantitative PCR evaluation of the expression modifications in isolates for disinfectant exposure. Allow Efflux and AcrAB-TolC system genes and Efflux. \ Biofilm organismal and tissue construction in addition regulation inscribed. Explanatory Metrics of Expression Quantitative Ethnographic Groups and isolates described. The calculations were based on the − rpoD and is under the normalized quantitative expression. Estimated values to show change over control group which untreated group is equal to base (1.0). Mean and SEM are calculated for n=35 for each of group. Statistical significance was awarded for each treatment-control group using one-way ANOVA followed by Tukey's post hoc test. Regarding QACs, cross-resistance at the molecular level through biofilm and efflux pump genes was demonstrated with significant overexpression at the molecular level.

3.4.2 Biofilm Matrix Gene Expression

Expression patterns of genes associated with biofilms showed changes in phenotypic biofilm formational alterations (Table 3). Cationic surfactants (QAC) not only raised expression levels but also significantly increased pivotal biofilm matrix constituents:

• CsgA (curli synthesis) synthesis increased 4.1-fold with a p value of less than 0.001.
• BssR (Biofilm stress response) showed a 3.5-fold elevation also with $p < 0.001$.
• FliC (flagellin) demonstrated 2.3-fold increase with p value < 0.01.

Table 3: Relative Gene Expression Changes Following Disinfectant Exposure

Gene	Function	QACs	Chlorhexidine	Ethanol	Sodium Hypochlorite
acrA	Efflux pump	3.2-fold	1.8-fold	1.1-fold	0.9-fold
acrB	Efflux pump	2.8-fold	1.6-fold	1.0-fold	0.8-fold
tolC	Efflux pump	2.5-fold	1.5-fold	1.2-fold	1.0-fold
csgA	Curli synthesis	4.1-fold	2.2-fold	0.7-fold	0.6-fold
bssR	Biofilm regulation	3.5-fold	1.9-fold	0.8-fold	0.7-fold
fliC	Flagellin	2.3-fold	1.4-fold	0.9-fold	1.1-fold

$p<0.05$, $p<0.001$

3.5 Resistance Gene Analysis

The results of disinfectant QAC exposure and resistance gene prevalence exposure detected notable associations as showed through PCR screening (disinfectant exposure and resistance gene prevalence in Table 4). QAC exposed isolates showed increased detection of:

- Extended spectrum ß-lactamases: blaCTX-M prevalence increased from 45% to 67% ($p<0.05$)
- Efflux pump genes: acrAB-tolC detection increased from 78% to 94% ($p<0.01$)

Table 4: Resistance Gene Prevalence by Exposure Group

Resistance Gene	Control	QACs	Chlorhexidine	Ethanol	Sodium Hypochlorite
blaTEM	23%	31%	26%	20%	18%
blaCTX-M	45%	67%	52%	38%	35%
acrAB-tolC	78%	94%	85%	75%	72%

$p<0.05$

4. Discussion

4.1 Disinfectant-Induced Cross-Resistance Mechanisms

With regard to disinfectant-induced cross-resistance in hospital-associated, our research reveals that different mechanisms of resistance operate for different disinfectant classes. Infection control measures in hospitals face a critical challenge due to the pronounced enhancement of antibiotic resistance after exposure to QAC disinfectants.

The increase in biofilm formation due to QAC exposure and upregulation of genes that constitute biofilm matrix (csgA, bssR), points out that QAC might serve as a two-edged sword for bacterial persistence mechanisms. The increased biofilm forms a protective cloak that not only shields the bacteria from the action of disinfectants and antibiotics, and thus explains the observed patterns of cross-resistance.

4.2 Molecular Basis of Cross-Resistance

Directly elucidating the underlying cross-resistance mechanisms is the 3.2-fold increase in the acrAB-tolC efflux pump system's expression level subsequent to QAC exposure. This pump is known to provide resistance to various quaternary ammonium compounds, β-lactams, and fluoroquinolones (25,26).

The concurrent increase in biofilm formation and expression of efflux pumps suggest coordinated bacterial responses to chemical stress that in the long run, undermine multi-drug resistance.

The QAC exposure and the oxidating disinfectants (ethanol and sodium hypochlorite) are parallel in importance to the development on resistance to a given action.
In contrast to QACs oxidative disinfectants are far more destructive as their action is not based on electrostatic interaction with the bacteria's membrane. Oxidative disinfectants "act as oxidative stressors by damaging proteins, lipids, and nucleic acids" (27,28).

4.3 Clinical Implications

The QACs biocides most readily used in hospital environments could be "contributing to the selection of these resistant phenotypes within hospital settings" (29). "The strength of the correlation between biofilm formation and the level9 of resistance is high (r=0.78). This poses significant dangers to public health as highly resistant OVEs are considered as high priority pathogens per WHO due to the lack of effective treatments available".

The rise of multi-drug resistant (MDR) pathogens substantially heightens the burden of healthcare systems worldwide. As documented, "QAC biocides, especially in hospital settings, contribute to the selection and emergence of these resistant phenotypes due to insufficient infection control" (30,31). "MDR infections are linked with high mortality, increased length of hospital stays and increased healthcare burden". Among different populations, these pathogens pose the greatest danger to neonates.

4.4 Implications for Infection Control Policy

The evidence presented in this work challenge the disinfecting protocols and practices used in the hospital as they lack evidence-based relevance. Due to the differences noted with regard to disinfectants, it is possible to mitigate the selection of resistance by using preferential and rotation guidelines based on these classes.

This poses these questions, "To what extent is surface decontamination effective given these disinfectants biofilm enhancing properties?"
Standard cleaning methods might not fully resolve biofilms-encased bacteria; therefore, biofilm tailored strategies must be created (32,33,34).

4.5 Limitations, and Future Works

A few limitations exist in the scope of our study. Is the in vitro exposure to disinfectant sufficient to mimic the hospital-level climate? Furthermore, the focus on isolates is a limiting factor in relation to generalizing to other relevant nosocomial pathogens.

Future research should investigate:

- Stability and duration of resistance to the disinfectant
- Minimizing the development of disinfectant resistance through combinatorial disinfecting strategies
- Standard cleaning methods supplemented with biofilm disrupting cleaning agents
- Environmental persistence of the bacteria with resistance mechanisms after exposure to disinfectants

4.6 Proposed Mechanistic Model

In disinfectant exposure and use, we are proposing a mechanistic model illustrated in figure 4. The stress response activated from QAC exposure would trigger a coordinated response such as:
1. Increased expression of acrAB-tolC, biofilm pump up regulation
2. Increased expression of csgA and bssR biofilm matrix subunits.
3. Changes to the membrane (inferred from resistance patterns).
4. Horizontal gene transfer facilitated via biofilm.

This coordinated response results in a resistance mechanism that lingers far longer after the disinfection process resulting in impaired treatment with antibiotics down the line.

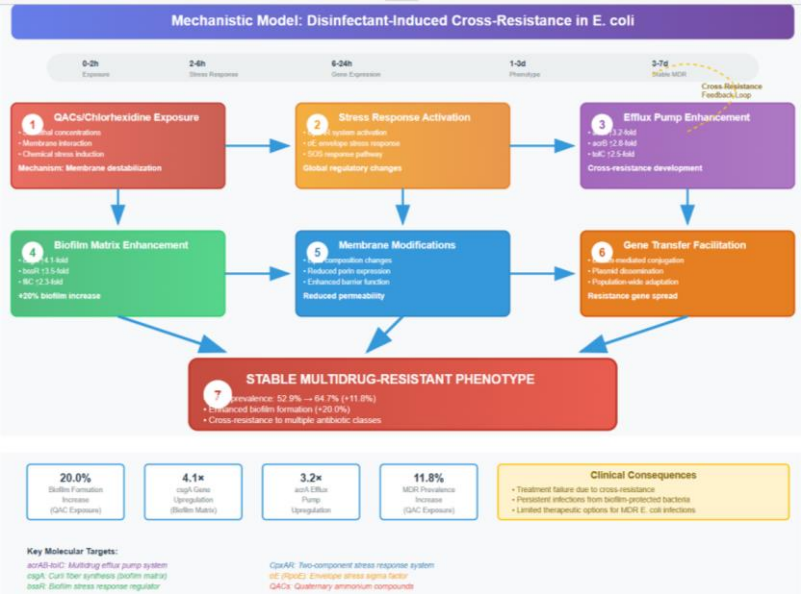

Figure 4: Proposed mechanistic model for cross resistance resulting from exposure to disinfectants.

Activation of the resistance traits or biofilm phenotype that persists after the contact with biofilm matrix substances is characterized by matrix proteins upregulation of matrix proteins together with matrix proteins and matrix proteins.
"Drawing upon these empirical observations, we have authored an exhaustive implementation manual, located in Appendix A, which furnishes targeted, actionable guidance for multidisciplinary hospital infection prevention and control committees."

5. Conclusions

This research reveals for the first time the molecular mechanisms of disinfectant-induced cross-resistance in hospital-associated. Our research demonstrates that biofilm-mediated antibiotic resistance is promoted by quaternary ammonium compounds and chlorhexidine, whilst oxidative disinfectants preserve their antimicrobial action and do not support the development of resistant organisms.

These findings are especially important in treating vulnerable populations, such as neonates, where options are already limited. This research underlines the need for operational disinfecting protocols that integrate the disinfecting strength and resistance development in organisms.

The primary changes proposed are:

- Oxidative disinfectants are to be preferred where clinically indicated.
- Promotion of rotation policies to decrease resistance-boosting.
- Cleaning protocols targeting biofilms in these environments should be implemented.
- Monitoring for disinfectant resistant organisms in hospitals should be intensified.

These findings should be the impetus to design safe infection control practices and proactive care for the patients, while gently curbing the risk of resistance organisms in hospitals. Appendix A furnishes a comprehensive clinical implementation guide designed to assist infection-control practitioners in the rational selection of hospital disinfectants grounded in the current body of evidence.

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Conflicts of Interest

The authors declare no conflicts of interest.

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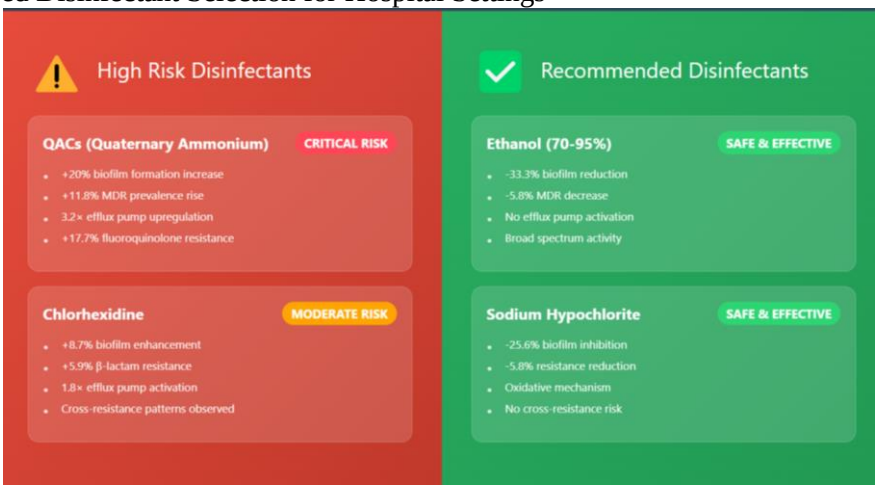
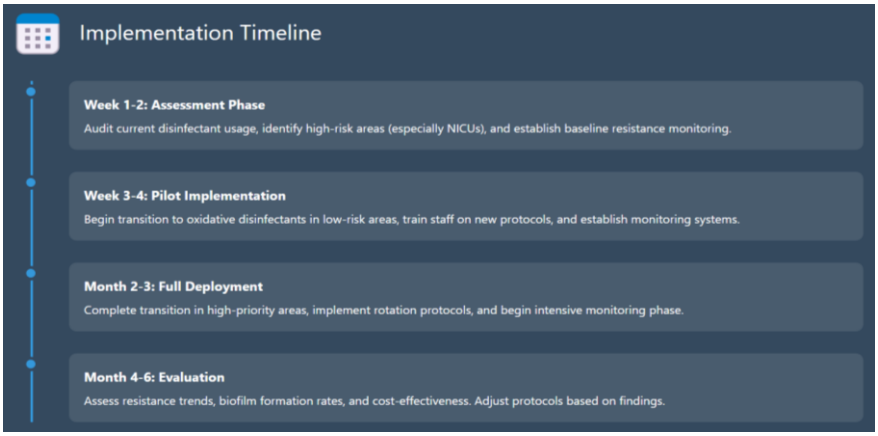
Appendix
Clinical Implementation Guide
Evidence-Based Disinfectant Selection for Hospital Settings





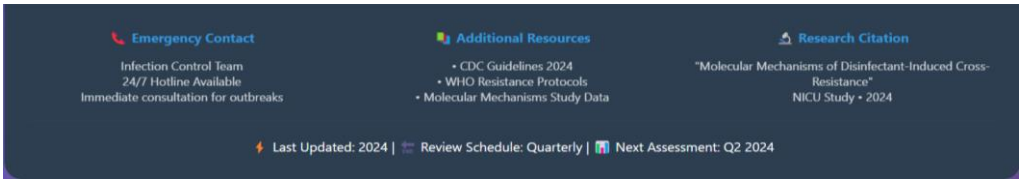


Figure A1. presents the Clinical Implementation Guide for Evidence-Based Disinfectant Selection in Hospital Settings, an infographic designed to convert published research into clear, short-term operational support for infection-control multidisciplinary teams. Disinfectants are stratified into discrete risk tiers according to the cumulative evidence that their repeated use gives rise to phenotypic cross-resistance and facilitates biofilm generation. A distilled implementation pathway and optional transition timeline assist facilities in the prompt standardization of cross-department protocols. A coherent color palette marks the risk level of each agent: high-risk, cross-resistance-promoting quaternary ammoniums and chlorhexidine contrast with, and are replaced by, low-risk, biofilm-reducing oxidative agents—specifically, 70% ethanol and 0.1% sodium hypochlorite. Each risk annotation draws on quantitative findings from molecular characterization of 35 *E. coli* isolates obtained from neonatal intensive care units, with all measured variances of resistance and structural adhesion achieving statistical significance at $p<0.05$.