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Differences in the Effects of Chlorhexidine on Optical Density and Colony Count Between Carbapenem-Sensitive and Carbapenem-Resistant Acinetobacter baumannii Strains

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ABSTRACT

Acinetobacter baumannii is a significant nosocomial pathogen with increasing carbapenem resistance. Chlorhexidine is a widely used antiseptic, but its efficacy against carbapenem-resistant strains remains unclear. This study aimed to compare the effects of chlorhexidine on optical density (OD) and colony count between carbapenem-sensitive and carbapenem-resistant *A. baumannii* strains. An experimental laboratory study was conducted using *A. baumannii* strains (sensitive and carbapenem-resistant). The strains were exposed to chlorhexidine (0.25% and 0.5%) for 20–120 seconds. OD was measured spectrophotometrically, and colony counts (CFU/mL) were determined after incubation. Data were analyzed using the Kruskal-Wallis test. Carbapenem-resistant strains showed higher tolerance to chlorhexidine, with slower reductions in OD and colony counts compared to sensitive strains. Significant differences were observed in colony counts after exposure to 0.5% chlorhexidine for ≥60 seconds ($p < 0.05$). OD values were less sensitive to exposure duration but varied significantly with concentration ($p < 0.05$). Chlorhexidine remains effective against carbapenem-resistant *A. baumannii*, but higher concentrations (0.5%) and longer exposure times (≥60 seconds) are required for optimal eradication. These findings support tailored antiseptic protocols in clinical settings with high resistance prevalence.

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INTRODUCTION

Hospital-acquired infections (HAIs) remain one of the most critical challenges in global healthcare systems, contributing to high morbidity, mortality, and prolonged hospital stays (Ali Aibalawy et al., 2024; Melariri et al., 2024; Sunarti, 2024). Among the pathogens responsible for HAIs, *Acinetobacter baumannii* has emerged as a major concern due to its remarkable ability to survive in hospital environments and its resistance to multiple antibiotics (Ahuatzin-Flores et al., 2024; Ibrahim et al., 2021; Nguyen & Joshi, 2021). This opportunistic Gram-negative bacterium frequently causes ventilator-associated pneumonia, bloodstream infections, urinary tract infections, and wound infections, especially among patients in intensive care units (ICUs) (Assefa, 2022; Fernández-Martínez et al., 2022; Halat & Moubareck, 2024). Its persistence in clinical settings and ability to form biofilms on medical devices make it particularly difficult to eradicate (Weber et al., 2023).

The rise of carbapenem-resistant *A. baumannii* (CRAB) represents a serious public health threat (Medioli et al., 2022; Sharma et al., 2021; Thacharodi et al., 2024). The World Health

Organization (WHO) has categorized CRAB as a "critical priority pathogen" because carbapenems, which are often used as antibiotics of last resort, are becoming increasingly ineffective against this organism. Resistance mechanisms such as β-lactamase production, efflux pump overexpression, and changes in membrane permeability allow CRAB to survive even in the presence of high concentrations of antimicrobial agents (Elshobary et al., 2025). Consequently, clinicians face limited therapeutic options, leading to poor clinical outcomes and higher mortality rates among infected patients (Giamarellou & Karaiskos, 2022; Shields et al., 2023).

In infection prevention and control, antiseptics play an essential role as a first-line defense to reduce microbial contamination on the skin and medical surfaces (Michael & Nguyen, 2022; Yang, 2024). Chlorhexidine, a bisbiguanide compound, is widely recognized for its broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and some viruses (Rahn et al., 2024). It has been extensively used in hand hygiene, preoperative skin preparation, and wound disinfection due to its ability to disrupt bacterial cell membranes and precipitate cytoplasmic contents. However, recent studies have raised concerns regarding the reduced susceptibility of multidrug-resistant organisms, including CRAB, to chlorhexidine (Fanelli et al., 2024; Huang et al., 2023).

Evidence suggests that CRAB may exhibit a higher tolerance to chlorhexidine exposure, potentially linked to genetic factors such as AdeABC efflux pump systems and alterations in outer membrane proteins (Wand et al., 2022). These mechanisms can decrease intracellular accumulation of chlorhexidine, resulting in reduced bactericidal activity (Ruan et al., 2023). The emergence of antiseptic tolerance among CRAB strains could undermine infection control programs that rely heavily on chlorhexidine-based protocols, particularly in ICU environments with high bacterial load and frequent antiseptic use. Therefore, understanding how CRAB responds to different concentrations and exposure durations of chlorhexidine is crucial for optimizing disinfection strategies (Fernandes et al., 2024).

Despite the extensive application of chlorhexidine in clinical practice, data comparing its effectiveness between carbapenem-sensitive and carbapenem-resistant *A. baumannii* strains remain limited (Elwakil et al., 2023; Eslami et al., 2025). Most existing research focuses on antibiotic susceptibility, while few studies have quantitatively assessed the impact of chlorhexidine on bacterial viability using indicators such as optical density (OD) and colony-forming unit (CFU) counts. Such comparative analyses are necessary to determine whether standard disinfection protocols are adequate against CRAB or if adjustments in concentration and exposure time are required to achieve optimal eradication.

This study was designed to evaluate and compare the effects of chlorhexidine on both carbapenem-sensitive and carbapenem-resistant *A. baumannii* strains using two key parameters: optical density and colony count. The primary objective of this research is to determine whether CRAB exhibits reduced susceptibility to chlorhexidine compared to carbapenem-sensitive strains and to identify the optimal concentration and exposure time required for effective bacterial eradication. By integrating optical density measurements—which provide a rapid assessment of bacterial turbidity and metabolic activity—with colony count enumeration that offers a direct

quantitative measure of viable microorganisms, this study aims to present a comprehensive understanding of chlorhexidine efficacy against different bacterial resistance profiles. The anticipated benefits of this research include providing evidence-based data to inform infection control policies, particularly in intensive care units and high-risk hospital environments where CRAB prevalence is high. Furthermore, the findings are expected to contribute to the optimization of antiseptic protocols by identifying specific concentration thresholds and exposure durations necessary to achieve maximal bactericidal effects, thereby reducing the risk of cross-transmission and supporting improved patient safety outcomes.

Ultimately, the findings of this study are expected to provide evidence-based insights for improving antiseptic use in healthcare facilities, especially in high-risk environments such as ICUs. By identifying the optimal concentration and exposure time of chlorhexidine required to effectively inactivate CRAB, this research contributes to infection control policies that minimize the risk of cross-transmission and support better patient safety outcomes. Furthermore, the study underscores the importance of continuous surveillance of antiseptic tolerance as part of a broader antimicrobial stewardship framework.

METHOD

This study employed an experimental laboratory design to investigate and compare the effects of chlorhexidine on *Acinetobacter baumannii* strains with different carbapenem susceptibility profiles. The research was conducted in the microbiology laboratory using two bacterial groups, namely carbapenem-sensitive *A. baumannii* and carbapenem-resistant *A. baumannii* (CRAB) isolates. Both strains were obtained from clinical specimens collected from hospitalized patients and were identified through standard microbiological methods, including Gram staining, biochemical testing, and antibiotic susceptibility testing using the Kirby-Bauer disk diffusion method. Carbapenem resistance was confirmed by reduced inhibition zones against imipenem and meropenem, following the Clinical and Laboratory Standards Institute (CLSI) guidelines.

The experimental procedure involved exposing each bacterial isolate to chlorhexidine at two different concentrations—0.25% and 0.5% (in 72% alcohol)—across various exposure durations of 20, 30, 60, 90, and 120 seconds. The control group consisted of bacterial suspensions that were not exposed to chlorhexidine. Prior to exposure, bacterial cultures were adjusted to a standard turbidity equivalent to 0.5 McFarland, ensuring consistent bacterial density across all test groups. After exposure to chlorhexidine, aliquots were neutralized using sterile phosphate-buffered saline (PBS) to halt the antiseptic action before further measurements.

To evaluate the impact of chlorhexidine on bacterial viability, two main parameters were measured: optical density (OD) and colony count (CFU/mL). The OD of each bacterial suspension was determined using a spectrophotometer at a wavelength of 600 nm, providing a rapid and quantitative indication of bacterial turbidity. For colony enumeration, samples were serially diluted and plated on nutrient agar, followed by incubation at 37°C for 18–24 hours. The resulting colonies

were counted manually and expressed as colony-forming units per milliliter (CFU/mL), allowing a direct comparison of viable bacterial populations before and after chlorhexidine exposure.

Data collection was performed in triplicate for each concentration and exposure time to ensure accuracy and reproducibility. The mean and standard deviation of OD and CFU values were calculated for both carbapenem-sensitive and carbapenem-resistant strains. Statistical analyses were conducted using the Kruskal–Wallis test, a non-parametric method suitable for comparing multiple independent samples that do not follow a normal distribution. A significance level of $p < 0.05$ was applied to determine whether differences between treatment groups were statistically significant.

The research workflow followed strict aseptic techniques to avoid contamination and ensure data reliability. All laboratory instruments, including pipettes, culture tubes, and spectrophotometers, were sterilized and calibrated before use. Ethical approval for the use of bacterial isolates was obtained from the institutional research ethics committee, as all isolates originated from anonymized clinical samples without patient identifiers.

By employing this rigorous methodological framework, the study ensured that variations observed in bacterial optical density and colony count were attributed solely to the effects of chlorhexidine exposure. This experimental approach allows for a robust evaluation of the comparative tolerance between carbapenem-sensitive and carbapenem-resistant *A. baumannii*, offering valuable insights for infection control practices and antiseptic optimization in hospital environments.

RESULTS AND DISCUSSION

The experimental results demonstrated notable differences in the response of **carbapenem-sensitive *Acinetobacter baumannii*** and **carbapenem-resistant *A. baumannii* (CRAB)** strains to chlorhexidine exposure across different concentrations and exposure times. The optical density (OD) values measured at 600 nm indicated that both strains experienced a decrease in turbidity following chlorhexidine exposure, suggesting a reduction in bacterial viability. However, the rate and extent of OD reduction were significantly greater in the carbapenem-sensitive strain compared to the CRAB isolate. At a concentration of **0.25% chlorhexidine**, OD reduction occurred gradually over time, while at **0.5%**, a sharp decline was observed within 60 seconds of exposure, indicating stronger bactericidal activity at higher concentration levels.

Quantitative colony count analysis further confirmed the differences in chlorhexidine susceptibility between the two strains. The carbapenem-sensitive strain exhibited a rapid decline in colony-forming units (CFU/mL) after 30 to 60 seconds of exposure to 0.5% chlorhexidine, with complete eradication achieved at 60 seconds. In contrast, the CRAB strain displayed a more gradual reduction in CFU values and required at least **120 seconds of exposure to 0.5% chlorhexidine** to achieve a comparable bactericidal effect. Statistical analysis using the **Kruskal–Wallis test** revealed a significant difference ($p < 0.05$) in CFU reduction between the two strains, particularly at the 0.5% concentration and longer exposure durations. These findings indicate that

CRAB has developed a higher tolerance to chlorhexidine compared to the carbapenem-sensitive strain.

The differences in chlorhexidine susceptibility observed in this study may be attributed to several **biological and molecular mechanisms** inherent to CRAB. Previous studies have shown that resistance in *A. baumannii* is often associated with **overexpression of efflux pump systems**, such as *AdeABC*, *AdeIJK*, and *AceI*, which actively expel antimicrobial compounds, including antiseptics like chlorhexidine, from the bacterial cell. Furthermore, **modifications in outer membrane proteins** and alterations in lipid composition may decrease cell membrane permeability, limiting chlorhexidine penetration and reducing its bactericidal activity. These adaptive mechanisms are consistent with CRAB's well-documented ability to survive under various environmental and chemical stresses.

From a microbiological perspective, the reduction in optical density represents changes in bacterial mass or turbidity rather than direct viability, while colony count provides a more precise measure of surviving bacteria. The observation that OD changes were less pronounced than CFU reductions suggests that chlorhexidine may initially affect bacterial metabolic activity and membrane integrity before leading to complete cell death. This two-phase action aligns with the **biphasic antimicrobial mechanism** of chlorhexidine, which involves rapid disruption of the bacterial membrane followed by precipitation of cytoplasmic components. Such findings support the notion that chlorhexidine remains an effective antiseptic agent but may require **adjusted exposure parameters** when used against highly resistant strains.

The results also highlight the importance of **concentration-dependent and time-dependent effects** of chlorhexidine. At lower concentrations (0.25%), the antiseptic effect was primarily bacteriostatic, temporarily inhibiting bacterial growth, whereas at 0.5%, it became bactericidal, resulting in complete cell death. This observation underscores the necessity of employing appropriate antiseptic concentrations in clinical disinfection protocols, particularly in high-risk hospital units such as ICUs, where CRAB contamination is prevalent. Using suboptimal concentrations could inadvertently promote tolerance and persistence of resistant strains on medical equipment and surfaces.

Comparing these findings with previous research, similar trends have been observed in studies conducted by **Akhmadi & Suprihadi** and **Loader**, which emphasized that higher antiseptic concentrations and prolonged exposure times significantly enhance the eradication of resistant bacterial populations. Moreover, the study by **Bagus** noted that mechanical factors, such as biofilm formation and surface adherence, also contribute to decreased antiseptic efficacy in *A. baumannii*. The consistency of the present results with these studies reinforces the validity of the observed chlorhexidine tolerance among CRAB isolates and underscores the need for tailored infection control strategies in healthcare environments.

In clinical application, these findings carry critical implications for **infection prevention and control (IPC)** practices. Chlorhexidine continues to serve as a cornerstone antiseptic in hospital disinfection protocols; however, the emergence of CRAB necessitates a re-evaluation of current usage standards. Implementing **higher chlorhexidine concentrations (0.5%)** and

ensuring **contact times of at least 60–120 seconds** can significantly improve decontamination outcomes in CRAB-endemic areas. Additionally, routine surveillance of antiseptic efficacy and monitoring for tolerance development should be incorporated into hospital IPC programs to prevent further resistance evolution.

In summary, the results of this study confirm that chlorhexidine retains its antimicrobial efficacy against both carbapenem-sensitive and carbapenem-resistant *A. baumannii*, but with differing kinetics of bacterial reduction. The CRAB strain demonstrates a higher level of tolerance, requiring greater exposure time and concentration to achieve equivalent bactericidal effects. These findings underscore the need for **evidence-based optimization of antiseptic protocols**, emphasizing concentration, exposure duration, and environmental monitoring as integral components of effective infection control in healthcare facilities.

CONCLUSION

This study demonstrated that chlorhexidine remains effective against both carbapenem-sensitive and carbapenem-resistant *Acinetobacter baumannii* (CRAB) strains, although CRAB requires higher concentrations and longer exposure times due to adaptive resistance mechanisms like efflux pump overexpression and altered membrane permeability. While carbapenem-sensitive strains were eradicated within 60 seconds at 0.5% chlorhexidine, CRAB needed at least 120 seconds of exposure to achieve similar bactericidal effects. These findings highlight the critical need to optimize chlorhexidine concentration and exposure duration, particularly in ICU and hospital settings with high CRAB prevalence, recommending 0.5% chlorhexidine for 60–120 seconds to prevent resistant subpopulation survival. Future research should investigate the molecular bases of antiseptic tolerance, including efflux pumps and biofilm formation, and conduct longitudinal surveillance of tolerance trends. Exploring combination therapies with other antimicrobials or physical methods may further improve efficacy, while translational studies are needed to evaluate the impact of optimized antiseptic protocols on healthcare-associated infection rates and patient outcomes.

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