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Short Communication

Chlorhexidine exposure of clinical *Klebsiella pneumoniae* strains leads to acquired resistance to this disinfectant and to colistin

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ABSTRACT

Chlorhexidine is widely used as a disinfectant in hospitals, which may impose a selective pressure on bacteria. This study aimed to determine whether continuous exposure to chlorhexidine could lead to adaptive resistance and cross-resistance as well as investigating potential resistance mechanisms. Three clinical *Klebsiella pneumoniae* strains susceptible to conventional antimicrobials were selected and were continuously cultured in broth with gradually increasing concentrations of chlorhexidine. Antimicrobial susceptibility was determined. Mechanisms of acquired resistance to chlorhexidine and colistin were analysed by PCR and reverse transcription quantitative PCR (RT-qPCR). Furthermore, fitness was assessed through growth curve assays. Increased resistance to chlorhexidine and colistin was observed in all strains. Expression of the *cepA* gene was upregulated in the adapted strains, suggesting that hyperexpression of CepA was probably the main mechanism of adaptive resistance to chlorhexidine. The amino acid substitutions Leu82Arg and Arg256Gly in PmrB were detected in all of the adapted strains, whilst Leu344Pro was only identified in one adapted strain, indicating that the PmrB substitution was responsible for the cross-resistant phenotype. Moreover, chlorhexidine adaptation might have an effect on bacterial growth.

1. Introduction

Klebsiella pneumoniae is a major nosocomial pathogen known to cause a variety of severe infections resulting in increased patient morbidity and mortality. Moreover, over the last few decades *K. pneumoniae* has rapidly developed multidrug resistance worldwide, limiting therapeutic choices [1]. Consequently, it is of particular importance to prevent the dissemination of *K. pneumoniae* in the clinical environment.

Chlorhexidine is a widely used disinfectant with broad-spectrum bactericidal activity. It was previously reported that 86–92% of skin flora was decreased by hand washing with chlorhexidine [2]. However, excessive use of chlorhexidine may impose a selective pressure and accelerate the emergence of chlorhexidine resistance. Bacteria with reduced chlorhexidine susceptibility have been reported [3]. The primary resistance mechanism to chlorhexidine in Gram-negative bacteria involves multidrug efflux pumps encoded by the *cepA*, *qacE* and *qacEΔ1* genes.

Long-term exposure of bacteria to chlorhexidine may also lead to cross-resistance to other antimicrobial agents with similar structures or mechanisms of action [4]. Undoubtedly there is an urgent need to understand the potential cross-resistance between chlorhexidine and antimicrobial agents as well as the molecular mechanisms involved. However, few reports on this subject are available at present. In the current study, three clinical *K. pneumoniae* isolates were cultured with gradually increasing concentrations of chlorhexidine to assess acquired resistance to this disinfectant as well as to other antimicrobial agents. Potential resistance mechanisms and fitness cost were also investigated.

2. Materials and methods

2.1. Bacterial strains

Three clinical isolates of *K. pneumoniae* (FK1891, FK2114 and FK2138) were recovered from three patients in different wards of the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) in 2015. The patients had not been exposed to chlorhexidine previously. The isolates were clonally unrelated [different pulsed-field gel electrophoresis (PFGE) patterns and

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sequence types] and were susceptible to almost all commonly used antibiotics. In addition, the isolates were also susceptible to chlorhexidine [5], with minimum inhibitory concentrations (MICs) of 8, 4 and 4 μg/mL, respectively.

2.2. Adaptation to chlorhexidine

The adaptation test was conducted as reported previously [6]. Briefly, 100 μL of an overnight culture (1×10^8 CFU/mL) was transferred to 10 mL of nutrient broth supplemented with $0.5 \times$ MIC of chlorhexidine. It was then incubated for 24-48 h and was further subcultured in broth containing the original concentration and a 2-fold higher concentration of chlorhexidine, respectively. Cultures grown in the higher chlorhexidine concentration following incubation were selected to repeat the above procedure. The process was continued until the strains reached their maximum tolerance. The stability of the adaptive tolerance was determined by repeated subculture in chlorhexidine-free broth for 10 generations.

2.3. Antimicrobial susceptibility testing

The MICs of chlorhexidine and commonly used antibiotics (colistin, nitrofurantoin, fosfomycin, chloramphenicol, tetracycline, gentamicin, aztreonam, meropenem, ciprofloxacin, ceftriaxone and cefepime) were determined by the agar dilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [7]. The MIC was defined as the lowest concentration of antibiotic or chlorhexidine that completely prevented bacterial growth, and a change of ≥4-fold in the MIC was considered significant. *Escherichia coli* ATCC 25922 was used as a control strain.

2.4. Detection of resistance determinants

The mechanisms of resistance to chlorhexidine and colistin were investigated according to the results of antimicrobial susceptibility testing. Total DNA from each of the *K. pneumoniae* strains was extracted using a Biospin Bacteria Genomic DNA Extraction Kit (Bioflux, Tokyo, Japan). Resistance genes to chlorhexidine (*cepA, qacE, qacEΔ1*) and colistin (*pmrA, pmrB, phoP, phoQ* and *mgrB*) were investigated by PCR and sequencing as described previously [8–10].

2.5. Reverse transcription quantitative PCR (RT-qPCR)

RNA of the *K. pneumoniae* strains was extracted using an RNeasy® Mini Kit (QIAGEN, Valencia, CA) and then was reverse transcribed using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). The expression level of the *cepA* gene was evaluated by RT-qPCR with forward (5′-TCCTGGTACGGCTGGCATCG-3′) and reverse (5′-GGTGATAATGTCCTGACGCTCCTC-3′) primers. All reactions were performed in triplicate. Relative expression of the *cepA* gene was normalised to that of the *gapA* reference gene and was determined by the $2^{-\Delta\Delta Ct}$ method [11].

2.6. Bacterial growth curves

Growth curve assays were conducted to assess the in vitro fitness of strains [10]. Briefly, overnight cultures of the strains were diluted with fresh Luria–Bertani broth at a final inoculum of 10^4 CFU/mL and were then cultured at 37°C. The optical density at 600 nm (OD_{600}) was measured every 2 h for 24 h. Experiments were repeated in triplicate and the mean absorbance values were used for analysis.

3. Results

3.1. Adaptation to chlorhexidine

Final MICs of chlorhexidine reached 128 μg/mL in all three strains following exposure to stepwise increasing concentrations of chlorhexidine. Furthermore, adaptive resistance to chlorhexidine was still stable following inoculation in chlorhexidine-free broth for approximately 10 passages.

3.2. Antimicrobial susceptibility testing

The MICs of a wide variety of antimicrobial agents were investigated to determine the presence of cross-resistance. Compared with wild-type strains, the adapted strains showed a remarkable increase in their resistance to colistin, with a change in MIC from 0.25 μg/mL to 32 μg/mL. However, no obvious change in susceptibility to other antimicrobial agents was observed following chlorhexidine exposure.

3.3. Molecular resistance mechanisms to chlorhexidine and colistin

The efflux pump gene *cepA* was identified in all of the strains, whilst *qacE* and *qacEΔ1* were not detected. The results of sequence analysis revealed the presence of point mutations in PmrB of the adapted strains compared with the wild-type strains. All of the adapted strains carried the mutations Leu82Arg and Arg256Gly. Moreover, the adapted strain of FK1891 also carried the point mutation Leu344Pro. Alterations in other proteins (PmrA, PhoP, PhoQ and MgrB) were not detected.

3.4. Analysis of cepA gene expression

All of the clinical strains showed an increased expression level of the *cepA* gene after adaptation to chlorhexidine. Compared with the wild-type strains of FK1891, FK2114 and FK2138, expression of *cepA* in their corresponding chlorhexidine-adapted strains was increased by 8.88-fold, 11.04-fold and 11.95-fold, respectively (Fig. 1).

3.5. Growth capacity of the wild-type and chlorhexidine-adapted strains

A similar growth trend was noted between the wild-type strains and their corresponding adapted strains (Fig. 2). All of the adapted strains showed a slightly lower growth rate in the initial 4 h. Strain FK1891 exhibited a statistically significant decrease in bacterial growth after chlorhexidine adaptation, whilst a contrary result was observed in strain FK2114 ($P < 0.05$). However, there was no statistically significant differences in growth of the wild-type and adapted strains of FK2138.

4. Discussion

Chlorhexidine is applied widely to prevent the spread of bacteria in the hospital environment. However, its extensive use has given rise to great concern about the development of resistance both to disinfectants and antibiotics owing to selective pressure. In a previous study, it was observed that bacteria recovered from patients with daily chlorhexidine bathing were more likely to have reduced chlorhexidine susceptibility than those from the patients without daily bathing [12]. Similar to previous reports [11], in the current study three clinical *K. pneumoniae* strains acquired stable resistance to chlorhexidine following repeated chlorhexidine exposure.

As well documented, the *cepA, qacE* and *qacEΔ1* genes are considered as important efflux mechanisms involved in chlorhexidine

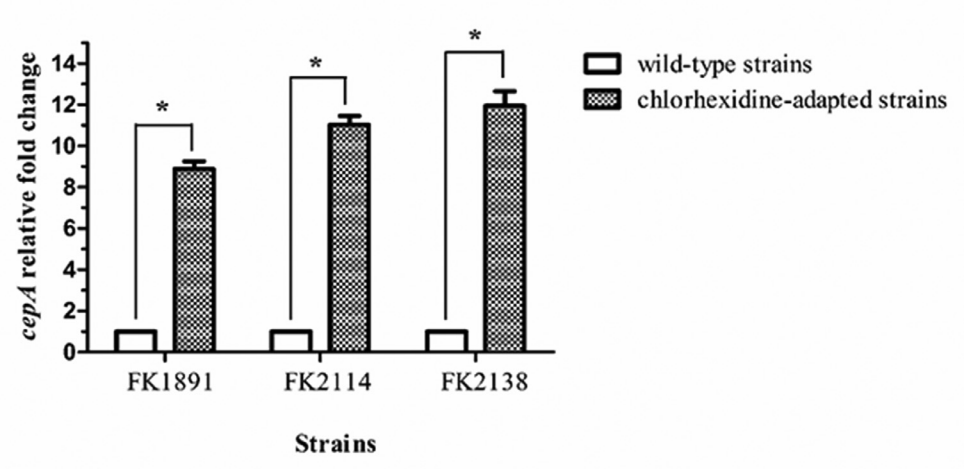

Fig. 1. Expression levels of the *cepA* gene in three *Klebsiella pneumoniae* strains. Bars and error bars indicate the mean values and standard deviation, respectively. * Significant difference in expression ($P < 0.05$).

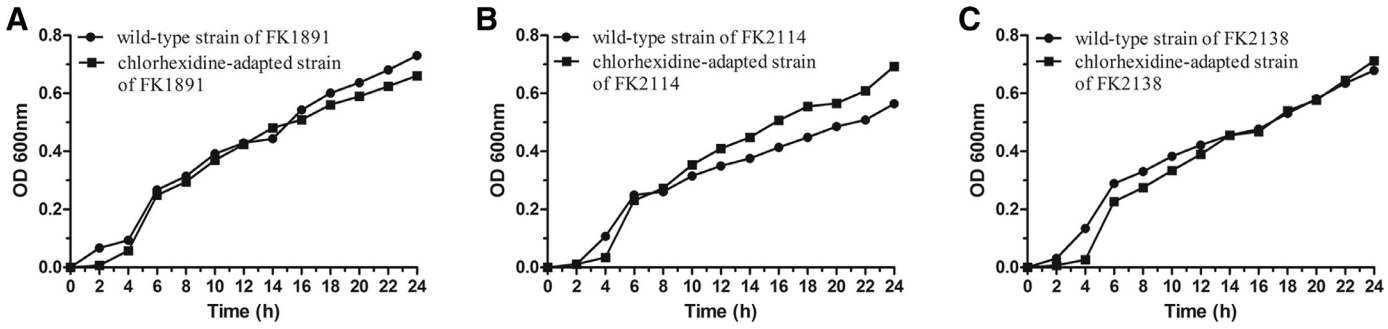

Fig. 2. Growth capacity of wild-type *Klebsiella pneumoniae* strains and their corresponding adapted strains. Data are the mean OD_{600} values of three independent experiments. OD_{600}, optical density at 600 nm.

resistance [13]. It has been reported that *cepA* was widely distributed among *K. pneumoniae* isolates with diverse chlorhexidine MICs [8]. All of the wild-type strains in the current study harboured the *cepA* gene, which was likely to be the basis for adaptive resistance. Similar to a previous report [13], upregulated expression of *cepA* was observed in the adapted strains, indicating that the active efflux pump contributed to transporting chlorhexidine out of the bacterial cell.

Russell et al. demonstrated that exposure of *Pseudomonas stutzeri* isolates to chlorhexidine resulted in cross-resistance to several antibiotics [14]. Similarly, it is worth noting that cross-resistance to colistin was observed in the current study. The MICs of colistin increased by 128-fold following chlorhexidine adaptation, posing a potential threat and further highlighting the necessity of prudent use of chlorhexidine.

Colistin, one of the most important last-resort antimicrobial agents, is used for the treatment of infections caused by multidrug-resistant bacteria and exhibits a relatively low resistance rate. Resistance to colistin is primarily due to modification of lipid A, mediated by changes in two-component systems such as PmrA/PmrB and PhoP/PhoQ, as well as by inactivation of MgrB. A previous study found that lipid A was altered in bacteria exposed to benzalkonium chlorides [15]. In the current study, cross-resistance to colistin was observed and the mutation Leu82Arg in PmrB associated with colistin resistance was found in all of the adapted strains. As previously reported, the mutation Arg256Gly was not linked to decreased susceptibility [16,17]. Thus, we speculated that Leu82Arg was probably the main colistin resistance mechanism, whilst another mutation Leu344Pro might not be related to colistin resistance since the latter mutation appeared not to confer an additional increase in MIC. However, further studies are still required to determine the effect of the mutation Leu344Pro. Strikingly, it is first reported that exposure to chlorhexidine caused mutations in PmrB associated with colistin resistance.

In accordance with a recent study performed with biocide-adapted bacterial strains [6], different growth capacities were found in the current study between the adapted strains and their respective wild-type strains, indicating that bacteria might change their physiological characteristics to adapt to the stress induced by chlorhexidine.

In conclusion, continued chlorhexidine exposure of bacterial strains significantly increased their resistance to chlorhexidine and colistin, primarily mediated by an active efflux pump encoded by *cepA* and a specific point mutation in PmrB, respectively. The findings of this study have important clinical significance, further emphasising the need for controlled use of chlorhexidine as well as constant surveillance of the chlorhexidine susceptibility of *K. pneumoniae* clinical strains.

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Competing interests

None declared.

Ethical approval

Not required.

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