Reduced susceptibility to chlorhexidine among extremely-drug-resistant strains of *Klebsiella pneumoniae*

L. Naparstek\textsuperscript{a, b}, Y. Carmeli\textsuperscript{a}, I. Chmelnitsky\textsuperscript{a}, E. Banin\textsuperscript{b}, S. Navon-Venezia\textsuperscript{a,*}

\textsuperscript{a}Molecular Epidemiology and Antimicrobial Resistance Laboratory, Division of Epidemiology, Tel Aviv Medical Centre, affiliated to the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

\textsuperscript{b}Mina and Everard Goodman Faculty of Life Sciences, Institute for Nanotechnology and Advanced Materials, Bar-Ilan University, Ramat Gan, Israel

**SUMMARY**

**Background:** Over the last decade, extremely-drug-resistant (XDR) strains of *Klebsiella pneumoniae* have emerged worldwide, mainly as a result of patient-to-patient spread. The predominant clone, sequence type 258 (ST258), is associated with high morbidity and mortality, and is a worldwide threat to public health. It was hypothesized that reduced susceptibility to chlorhexidine, the most widely used hospital disinfectant, may contribute to the endemic nature of this strain.

**Aim:** To characterize and compare the susceptibility of the epidemic *K. pneumoniae* clone ST258 and non-epidemic *K. pneumoniae* clones to chlorhexidine.

**Methods:** The minimum inhibitory concentration (MIC) of chlorhexidine was determined in 126 XDR *K. pneumoniae* clinical isolates using agar dilution. Expression of three different efflux pumps — *cepA*, *acrA* and *kdeA* — was investigated in the absence and presence of chlorhexidine using quantitative real-time polymerase chain reaction. Heteroresistance to chlorhexidine was identified using population analysis.

**Findings:** The MIC of chlorhexidine was higher for *K. pneumoniae* ST258 (*N* = 70) than other *K. pneumoniae* sequence types (*N* = 56); 99% of ST258 isolates had MICs > 32 \( \mu \text{g/mL} \), compared with 52% of other *K. pneumoniae* sequence types (*P* < 0.0001). Reduced susceptibility to chlorhexidine appeared to be independent of the expression of *cepA*, *acrA* and *kdeA* efflux pumps. Chlorhexidine-resistant subpopulations were observed independent of the bacterial sequence type or the MIC.

**Conclusions:** Reduced susceptibility to chlorhexidine may contribute to the success of XDR *K. pneumoniae* as a nosocomial pathogen, and may provide a selective advantage to the international epidemic strain *K. pneumoniae* ST258. The heterogeneous nature of chlorhexidine-resistant subpopulations suggests that this phenomenon might not be rendered genetically.

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**Introduction**

Over the last decade, extremely-drug-resistant (XDR), carbapenemase-producing strains of *Klebsiella pneumoniae* have emerged in the USA and other parts of the world.\textsuperscript{1,2} The
predominant clone has been identified as sequence type 258 (ST258); this was epidemic in the USA and spread to other countries mainly as a result of patient-to-patient spread. K. pneumoniae ST258 is resistant to almost all available antibiotics and is associated with high morbidity and mortality. As such, it represents a worldwide threat to public health.

ST258 emerged in Israel in 2006, causing a national outbreak. Previously, the authors described the co-existence of other carbapenemase-producing K. pneumoniae clones together with the appearance of K. pneumoniae ST258 in their institution. Although these clones share a common XDR profile that presumably offers similar selective advantages under selective antibiotic pressure, K. pneumoniae ST258 accounts for more than 95% of clinical isolates. Multiple reports from various geographical areas indicate the spread of this clone to other countries. The authors speculated that this strain has unique features that are advantageous for its persistence in the hospital environment.

Chlorhexidine is a topical antiseptic agent with a broad spectrum of activity, used extensively in hospitals in various applications such as surface cleaning, hand disinfection and skin preparation before invasive procedures. A possible advantage to a bacterium in the hospital setting would be reduced susceptibility to commonly used disinfectants.

This study aimed to examine the susceptibility of clinical isolates of XDR K. pneumoniae to chlorhexidine, and to compare ST258 with other XDR K. pneumoniae lineages.

Methods

Bacterial strains

One hundred and twenty-six XDR K. pneumoniae strains isolated from unique patients and various clinical sources by the Clinical Microbiology Laboratory of Tel-Aviv Sourasky Medical Centre were included in the study. These isolates had been genotyped previously and were assigned to one of two groups: Group I (N = 70) comprised ST258 carbapenemase-producing isolates; and Group II (N = 56) comprised non-ST258 isolates belonging to various clones and with various multi-drug-resistant phenotypes, including extended-spectrum beta-lactamase and carbapenemase production.

Testing the minimum inhibitory concentration of chlorhexidine

The minimum inhibitory concentration (MIC) of chlorhexidine was determined using agar dilution in accordance with the 2011 guidelines of the Clinical and Laboratory Standards Institute. Cation-adjusted Müller-Hinton (MH) agar containing 0–256 μg/mL chlorhexidine digluconate (Sigma, St. Louis, MO, USA) was used. K. pneumoniae ATCC13883 (MIC of chlorhexidine 16 μg/mL) and Escherichia coli ATCC25922 (MIC of chlorhexidine 2 μg/mL) were included in each experiment as control strains. Experiments were performed in triplicate, with consistent results.

Quantitative real-time polymerase chain reaction

The expression of three K. pneumoniae efflux pumps — cepA, acrA and kdeA — was determined in four isolates using quantitative real-time polymerase chain reaction (qRT-PCR): two with lower MICs of chlorhexidine (32 μg/mL) and two with higher MICs of chlorhexidine (128 μg/mL). K. pneumoniae ATCC13883 was included as a control strain. Total RNA was purified during logarithmic growth of each isolate grown in the absence and presence of subinhibitory chlorhexidine (4 μg/mL) using an RNAProtect and RNEasy minikit (QiAGEN, Duesseldorf, Germany) in accordance with the manufacturer’s instructions. cDNA was created using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA), and a three-step Syber-green fluorescence qRT-PCR reaction was performed (Rotor-Gene, Corbett Research, Sydney, Australia). Efflux pump expression was calculated relative to the expression of K. pneumoniae housekeeping gene rpoB using Rotor-Gene 6000 software (Version 1.7). RNA was purified from two biological repeats, and cDNA was created separately in duplicate for each RNA preparation. qRT-PCR experiments were performed on each cDNA sample in triplicate.

Population analysis

Population analysis experiments were carried out on the four K. pneumoniae isolates examined in the qRT-PCR experiments. An overnight culture in MH medium originating from a single colony of each isolate was serially diluted in saline and plated on to MH agar containing a range of concentrations of chlorhexidine (0–256 μg/mL). Experiments were performed in triplicate, and mean viable counts were plotted against the respective concentration of chlorhexidine. E. coli ATCC25922 served as a homogenous chlorhexidine-susceptible control strain. Homogeneity in susceptibility to chlorhexidine was defined using the common definition used for antimicrobials, where an increased concentration of chlorhexidine (up to the MIC) leads to minimal or no change in inhibition (colony-forming units/ml), whereas a sharp decrease is observed at concentrations equal to the MIC. Heterogeneity was defined as deviation from this pattern. To investigate more resistant subpopulations, an additional population analysis experiment was performed on an overnight culture originated from a single chlorhexidine-resistant colony that grew on MH agar containing 64 μg/mL chlorhexidine in the first population analysis experiment.

Statistical methods

The MICs of chlorhexidine for Group I and Group II isolates were compared using the Mann–Whitney test. Differences in MIC distribution were analysed by testing for equality of populations using the Kruskal–Wallis test. Efflux pump expression results were analysed using the unpaired t-test. Differences with a P-value of ≤0.05 were considered to be statistically significant.

Results

The MICs of chlorhexidine ranged from 8 to >256 μg/mL (mean 140 μg/mL), which were generally higher than those observed for K. pneumoniae ATCC13883 and E. coli ATCC25922 control strains (16 μg/mL and 2 μg/mL, respectively). The 70 ST258 isolates tested (Group I) showed a narrow distribution of higher MICs of chlorhexidine (32–256 μg/mL) compared with much wider distribution of generally lower MICs of
chlorhexidine among the 56 non-ST258 isolates (Group II) (8–256 µg/mL) (Figure 1). This difference in distribution was statistically significant ($P < 0.0001$). Ninety-nine percent of Group I strains had MICs of chlorhexidine of $>32$ µg/mL, compared with 52% of Group II strains ($P < 0.0001$).

In an attempt to understand the mechanism responsible for the differences in the distribution of MICs of chlorhexidine in Groups I and II, the RNA expression levels of three efflux pumps — cepA, acrA and kdeA — were quantified. It has previously been reported that the cepA efflux pump is associated with chlorhexidine tolerance in $K. pneumoniae$,$^{13}$ while acrA and kdeA are other major $K. pneumoniae$ efflux pumps. No correlation was found between the expression of any of the efflux pumps and the bacterial sequence type or MIC of chlorhexidine ($P = 0.19$) (Figure 2a). Pre-exposure to chlorhexidine led to a 1–1.5-fold increase in expression compared with expression in the absence of chlorhexidine for all four $K. pneumoniae$ isolates analysed (Figure 2b–d), while $K. pneumoniae$ ATCC13883 showed a 2–3-fold increase in the expression of all three efflux pumps under the same conditions. It was concluded that the expression of cepA, acrA and kdeA, as measured by RNA level, is not responsible for the high MICs of chlorhexidine observed.

The hypothesis that heterogeneity of chlorhexidine susceptibility might contribute to the high MICs of chlorhexidine observed among $K. pneumoniae$ ST258 isolates was also examined. Population analysis experiments were performed using four representative isolates. All four isolates (ST258, $N = 2$; ST340, $N = 1$; ST376, $N = 1$) demonstrated population heterogeneity with respect to MICs of chlorhexidine (Figure 3), whereby a more resistant subpopulation was observed within each population tested. This phenomenon was observed independently of sequence type or the overall population MIC of chlorhexidine. The nature of these less-susceptible subpopulations seemed to be transient, as population analysis performed on a single more-resistant colony (64 µg/mL chlorhexidine) showed a heterogeneous MIC of chlorhexidine similar to that of the original population (data not shown). The transient nature of these subpopulations ruled out the

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**Figure 1.** Distribution of the minimum inhibitory concentrations of chlorhexidine among 126 extremely-drug-resistant Klebsiella pneumoniae strains. Isolates belonging to the epidemic lineage ST258 (black bars, $N = 72$) were significantly less susceptible to chlorhexidine than other sequence types (grey bars, $N = 56$) ($P < 0.05$).

**Figure 2.** Expression of efflux pumps and the effect of subinhibitory concentrations of chlorhexidine. Expression of cepA, kdeA and acrA efflux pumps, as judged by quantitative real-time polymerase chain reaction (a), and the change in expression in the presence of chlorhexidine (b–d) are shown for four strains. The minimum inhibitory concentration (MIC) of chlorhexidine for Klebsiella pneumoniae (Kpn) 490 and Kpn588 was 128 µg/mL, and the MIC of chlorhexidine for Kpn103 and Kpn531 was 32 µg/mL. The expression level of the efflux pumps was independent of the bacterial sequence type or the MIC of chlorhexidine, and was unaffected by the presence of a subinhibitory concentration of chlorhexidine ($P > 0.05$). Black bars, cepA; grey bars, kdeA; white bars, acrA.
was 32 most frequently reported MIC, using the agar dilution method, susceptibility to chlorhexidine among this species, but the seem that any of these efflux pumps undergo overexpression in some of the tested isolates compared with other isolates or the pumps are responsible for the higher MICs of chlorhexidine in were present in all isolates independent of their MIC of chlorhex-ide. Reduced susceptibility to disinfectants among bacteria often involves the action of active or overexpressed efflux pumps. In K. pneumoniae, the cepA efflux pump is associated with reduced susceptibility to chlorhexidine, so this study examined its potential role along with that of two additional pumps — acrA and kdeA. As all three efflux pump genes were present in all isolates independent of their MIC of chlorhexidine or sequence type, the expression levels was also examined (Figure 2). The data suggested that none of these pumps are responsible for the higher MICs of chlorhexidine in some of the tested isolates compared with other isolates or the K. pneumoniae ATCC control strain. Furthermore, it does not seem that any of these efflux pumps undergo overexpression in the presence of a subinhibitory concentration of chlorhexidine (Figure 2b—d). Nevertheless, it is important to emphasize that the qRT-PCR findings reflect the total expression of each type of efflux pump tested in the whole bacterial population. As efflux pump expression of a less-susceptible subpopulation would not be detected using qRT-PCR, population analysis was performed to test the possibility of the presence of less-susceptible subpopulations within each isolate. The findings demonstrate the existence of tolerant subpopulations (Figure 3). Heteroresistance towards antibiotics has been described previously for other opportunistic pathogens such as Acinetobacter baumannii; however, to the authors’ knowledge, this is the first study to demonstrate population heterogeneity towards a disinfectant. The presumably transient nature of these subpopulations raises questions about the underlying mechanism; further investigation is required. Finally, the clinical relevance of higher MICs of chlorhexidine for K. pneumoniae ST258 should be considered in the context of the global threat of these extremely drug-tolerant strains. It is possible that the resistance of this strain to chlorhexidine contributes to its ability to persist in the hospital environment.

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Conflict of interest statement
None declared.

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