*Journal of Hospital Infection* (2002) **51**: 106–113
doi:10.1053/jhin.2002.1204, available online at http://www.idealibrary.com on IDEAL®

# Antibiotic resistance as an indicator of bacterial chlorhexidine susceptibility

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**Summary:** The antibiotic and chlorhexidine (CHX) susceptibility of 70 distinct clinical isolates: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus* (not MRSA), *Streptococcus pyogenes* and *Enterococcus faecalis* (10 of each) were tested using minimal bactericidal (MBC) and/or minimal inhibitory (MIC) concentrations. Non-fermentative bacteria tolerated CHX at high concentrations; Gram-positive cocci, especially *S. pyogenes*, were the most susceptible. We found a good correlation between CHX and antibiotic susceptibility in both MIC and MBC among Gram-negative bacteria, and mainly in MBC among Gram-positive bacteria. Resistance to ciprofloxacin, imipenem, cefotaxime, ceftazidime, gentamicin and aztreonam appeared to indicate increased CHX resistance among Gram-negative bacteria. This finding gives clinicians the ability to predict CHX susceptibility according to routine antibiotic resistance testing.



*Keywords:* Chlorhexidine; antibiotic resistance; biocides; disinfectants; nosocomial infection.

## Introduction

During the last decades, a dramatic increase in hospital-acquired infections caused by multi-drug resistant microbes has taken place. As a result, the threat of microbial contamination and infection has led to an increased use of disinfectants and antiseptics.[1] Nevertheless, during hospital outbreaks, the environmental spread of antibiotic-resistant pathogens has been demonstrated.[2,3] Moreover, nosocomial pathogen isolates from the hospital environment are shown to be particularly resistant to antibiotics.[4,5]

Chlorhexidine (CHX) is a biguanide compound, widely used in clinical practice as a skin and mucous membrane antiseptic and a disinfectant. Local CHX treatment has proved to be effective in the prevention of nosocomial respiratory tract infections, urinary tract infections in transurethral surgery, reducing maternal and neonatal infectious morbidity during childbirth, and decreasing mortality in experimental intra-abdominal sepsis.[6–10]

However, bacterial resistance to CHX has been detected,[11,12] but, there is a lack of a simple method for routine testing of CHX susceptibility. The possibility of predicting CHX susceptibility by routine antibiotic susceptibility profiling needs to be addressed.

Contradicting data have been presented about the correlation of biocide and antibiotic resistance. Irizarry *et al.*[13] and Suller *et al.*[14] showed methicillin-resistant *Staphylococcus aureus* (MRSA) strains to be less susceptible than methicillin-sensitive *S. aureus* (MSSA) to CHX and quaternary ammonium compounds (QAC). In contrast, Al-Masaudi *et al.*[15] have described MRSA and MSSA as equally sensitive to CHX, phenols and esters but MRSA strains were more resistant to QAC. On the other hand, in some experiments, no differences between CHX, triclosan and povidone iodine susceptibility of MRSA and MSSA strains were found.[1,16,17] In addition, the biocide

Received 22 May 2001; revised manuscript accepted 12 February 2002; published online 22 May 2002.
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susceptibility of vancomycin-resistant and sensitive enterococci has been found to be similar both in terms of MIC testing and time-kill studies.[14,17] In Gram-negative bacteria, a correlation between antibacterial multi-resistance and MICs of cationic antiseptics has been found.[18] The available information about the linkage of biocide and antibiotic resistance is mainly focused on determining resistance among one or few bacterial species. Consequently, existing data are not sufficient for empirical application of antiseptics, including CHX.

The aim of our study was to compare the CHX and antibiotic susceptibility patterns of various bacteria in order to elucidate if antibiotic susceptibility can be used as the marker of presumable biocide activity.

## Materials and methods

Seventy consecutive distinct clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus* (not MRSA), *Streptococcus pyogenes* and *Enterococcus faecalis* (10 of each) from the Laboratory of Microbiology, Tartu University Clinics, were investigated. Bacteria were identified according to standard microbiological procedures.

The minimal inhibitory concentration of each antibiotic was determined by E-tests (AB Biodisk, Solna, Sweden) according to the manufacturer. Antibiotics tested were: piperacillin, cefotaxime, ceftazidime, cefepime, piperacillin/tazobactam, aztreonam, imipenem, gentamicin, amikacin and ciprofloxacin for Gram-negative bacteria; methicillin, amoxycillin, cefalothin, cefuroxime, amoxycillin/clavulanic acid, gentamicin, clarithromycin and clindamycin for *S. aureus* and *S. pyogenes*; and ampicillin, gentamicin and erythromycin for *E. faecalis*. The interpretation of the MIC was performed according to National Committee for Clinical Laboratory Standards.[19] Bacterial susceptibility to chlorhexidine digluconate (Oriola, Espoo, Finland) was estimated using a macrodilution method (NCCLS[20]). Briefly, a concentration of approximately $5 \times 10^5$ of bacterial cultures was inoculated into two-fold serial dilutions (0.125–512 mg/L) of CHX in Mueller-Hinton broth for MIC detection. Subcultures from tubes showing inhibition of growth were performed for the MBC estimation.

Statistical analysis was performed with Jandel SigmaStat 2.0 software using Spearman's correlation test (correlation of MIC and MBC values) and Mann-Whitney's rank sum test (comparison of groups).

## Results

The antibiotic susceptibility patterns of tested microbes are shown in Table I. 36.7% (11/30) of Gram-positive bacteria and 22.5% (9/40) of Gram-negative bacteria were fully susceptible to all tested antibiotics. No fully resistant bacterial isolates were detected.

Macrodilution assay showed the growth inhibition of the tested microbes at CHX concentrations from 0.25 to 128 mg/L. The range of CHX MIC and CHX MBC values was wider in Gram-negative bacteria. For Gram-negative bacteria, CHX MIC values varied from 1 to 64 mg/L and for Gram-positive bacteria from 0.25 to 8 mg/L. Gram-negative bacteria showed CHX MBC values from 1 to 128 mg/L and Gram-positive bacteria from 1 to 32 mg/L. However, in individual Gram-positive strains, MIC and MBC values differed significantly more than among the Gram-negative ones. There was four or more times difference between MIC and MBC values among 66.7% of Gram-positive and 37.5% of Gram-negative isolates ($P = 0.03$). Positive correlation between CHX MIC and CHX MBC values both in Gram-positive and Gram-negative bacteria (correlation coefficient 0.735 and 0.569, $P < 0.001$ and $P = 0.04$, respectively) was found.

CHX susceptibility, in terms of both MIC and MBC, was species and cell wall structure specific. In general, Gram-positive bacteria were more susceptible to CHX than Gram-negative bacteria (Figure 1). We found differences between Gram-positive and Gram-negative bacteria concerning MIC median (1.0 and 16.0 mg/L, respectively; $P < 0.001$) and MBC median (6.0 and 32.0 mg/L, respectively; $P < 0.001$) values. The only exception was *E. faecalis* showing an even higher CHX MBC value than the most susceptible Gram-negative bacteria, particularly *E. coli* (MBC median values 24.0 and 6.0 mg/L, $P = 0.009$). Statistically important differences of CHX MIC and MBC values found between bacterial species are presented in Table II (a and b). Among Gram-negative bacteria, both MIC and MBC of *E. coli* differed statistically from other species. In the Gram-positive group, the same tendency was seen concerning *E. faecalis*.

Among Gram-negative bacteria, the species most susceptible to CHX was *E. coli* and among

**Table I** *The antibiotic susceptibility patterns of tested microbes (N = 70)*

| | *S. aureus* | | *S. pyogenes* | | *E. faecalis* | | *E. coli* | | *K. pneumoniae* | | *P. aeruginosa* | | *A. baumannii* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC* | S %# | MIC | S % | MIC | S % | MIC | S % | MIC | S % | MIC | S % | MIC | S % |
| Methicillin | 0.75 | 100 | 0.158 | 100 | | | | | | | | | | |
| Ampicillin | | | | | 0.75 | 100 | | | | | | | | |
| Amoxicillin | 13.5 | 50 | 0.016 | 100 | | | | | | | | | | |
| Piperacillin | | | | | | | 1.5 | 80 | 256 | 40 | 16 | 70 | 256 | 20 |
| Piperecillin/ Tazobactam | | | | | | | 1.5 | 100 | 4 | 70 | 14 | 80 | 224 | 10 |
| Amoxicillin/ Clavulanic acid | 0.75 | 100 | 0.016 | 100 | | | | | | | | | | |
| Cefalothin | 0.38 | 100 | 0.19 | 100 | | | | | | | | | | |
| Cefuroxime | 1.5 | 100 | 0.016 | 100 | | | | | | | | | | |
| Cefotaxime | | | | | | | 0.056 | 100 | 9 | 50 | 192 | 20 | 256 | 10 |
| Ceftazidime | | | | | | | 0.19 | 100 | 40 | 40 | 3 | 80 | 24 | 10 |
| Cefepime | | | | | | | 0.047 | 100 | 1 | 100 | 7 | 80 | 192 | 60 |
| Aztreonam | | | | | | | 0.064 | 100 | 80 | 40 | 10 | 50 | 80 | 10 |
| Imipenem | | | | | | | 0.19 | 90 | 0.22 | 100 | 6 | 50 | 0.875 | 90 |
| Gentamicin | 0.315 | 100 | 4 | 60 | 12 | 60 | 0.625 | 90 | 12 | 20 | 3 | 100 | 16 | 20 |
| Amikacin | | | | | | | 1.75 | 100 | 1.5 | 90 | 7 | 100 | 16 | 60 |
| Ciprofloxacin | | | | | | | 0.014 | 100 | 0.148 | 90 | 0.69 | 80 | 9 | 20 |
| Erythromycin | | | | | 256 | 10 | | | | | | | | |
| Claritromycin | 0.036 | 90 | 0.032 | 100 | | | | | | | | | | |
| Clindamycin | 0.064 | 100 | 0.079 | 100 | | | | | | | | | | |

*MIC, medians of minimal inhibitory concentration (mg/L); #S%, percentage of strains fully susceptible.

Gram-positive bacteria *S. pyogenes* [Figure 2(a, b)]. Non-fermentative bacteria, *P. aeruginosa* and *A. baumannii* showed the highest CHX MIC and CHX MBC values. The variability of CHX MIC was largest among *A. baumannii* isolates also showing the highest CHX MIC median values (range 8–64 mg/L, MIC median 32 mg/L). *P. aeruginosa* was the most variable in CHX MBC values and also showed the highest CHX MBC median values (range 32–128 mg/L, MBC median 64 mg/L).

The correlation between MIC of investigated antibiotics and CHX susceptibility values for Gram-positive and Gram-negative bacteria are shown in Table III. Among Gram-negative bacteria, there was a significant positive correlation between MIC of antibiotic and CHX MIC and MBC values to all investigated antibiotics. The strongest correlation was seen in ciprofloxacin, imipenem and piperacillin. However, for Gram-positive bacteria, this correlation occurred mainly with CHX MBC values and not for all antibiotics tested (Table III).

We found antibiotic resistance to give reliable information about CHX susceptibility of Gram-negative bacteria. Figure 3 shows CHX susceptibility of antibiotic-resistant vs. sensitive isolates. Statistically important differences were found in CHX susceptibility comparing resistant and sensitive isolates with the following antibiotics—imipenem, ceftazidime, cefotaxime, aztreonam, gentamicin and ciprofloxacin. Among Gram-positive bacteria, statistically significant differences between antibiotic resistance and CHX susceptibility were not found.

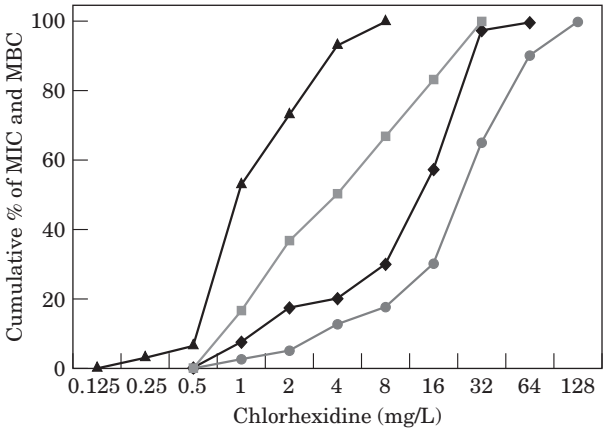


**Figure 1** Cumulative distribution of chlorhexidine MIC and MBC values (mg/L) of Gram-positive and Gram-negative bacteria. ▲, Gram-positive MIC; ◆, Gram-negative MIC; ■, Gram-positive MBC; ●, Gram-negative MBC.

**Table II** *(a) Comparison of CHX susceptibility values (medians of MIC/MBC) between Gram-negative bacteria (b) Comparison of CHX susceptibility values (medians of MIC/MBC) between Gram-positive bacteria*

| | Medians | | Statistical differences (*P*) MIC/MBC | | |
|---|---|---|---|---|---|
| Organism | MIC | MBC | *K. pneumoniae* | *A. baumannii* | *P. aeruginosa* |
| (a) | | | | | |
| *K. pneumoniae* | 16 | 32[a] | | | |
| *A. baumannii* | 32 | 32 | NS/NS | | |
| *P. aeruginosa* | 16 | 64 | NS/0.007 | NS/NS | |
| *E. coli* | 2 | 6[a] | <0.001/0.002 | <0.001/<0.001 | <0.001/<0.001 |
| (b) | | | | | |
| | | | *E. faecalis* | *S. aureus* | |
| *E. faecalis* | 4 | 24 | | | |
| *S. aureus* | 1 | 6 | <0.001/<0.001 | | |
| *S. pyogenes* | 1 | 1.5 | <0.001/<0.001 | NS/0.001 | |

NS, not significant. [a]MIC, MBC expressed in mg/L.

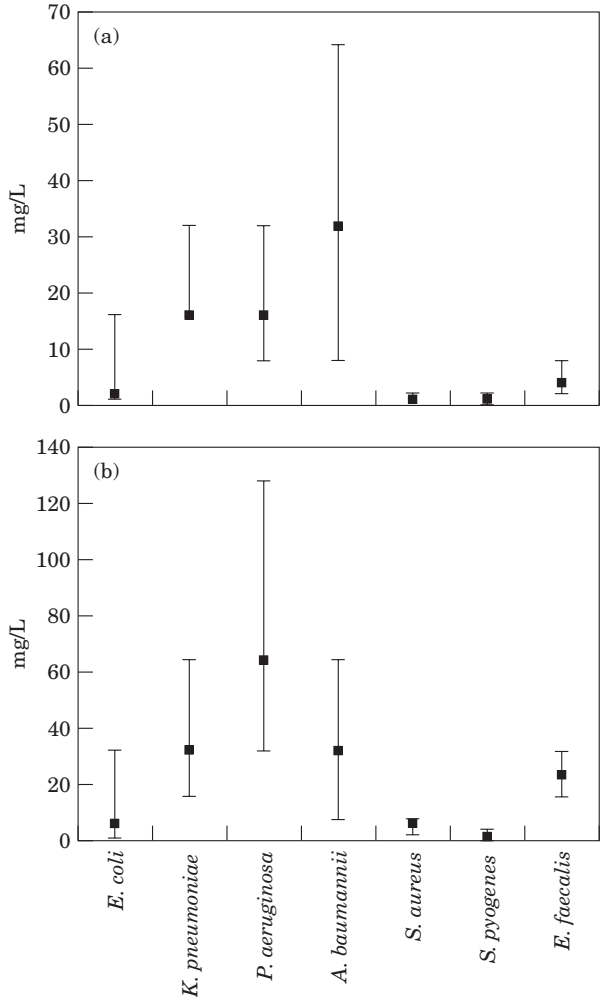


**Figure 2** Range and medians (■) of chlorhexidine MIC (a) and MBC (b) of tested microbes.

## Discussion

Our study showed a strong correlation between the CHX and antibiotic susceptibility of bacteria. Gram-positive bacteria were more sensitive to CHX than Gram-negative bacteria, and species specificity in CHX susceptibility could be detected.

In general, Gram-positive bacteria are considered more sensitive to disinfectants than Gram-negative bacteria due to the composition of the cell wall.[1] For both, Gram-positive and Gram-negative bacteria biocide resistance has been associated with intrinsic and acquired resistance mechanisms. Among acquired resistance mechanisms, plasmid-mediated low-level resistance of Gram-positive bacteria to cationic disinfectants, including CHX, has been shown.[21,22] However biocidal resistance of Gram-negative bacteria is mainly associated with intrinsic mechanisms and chromosomal mutations rather than plasmids.[23,24]

Several in vitro methods for studying biocide activity at different concentrations such as carrier tests and quantitative suspension tests[12,25,26] have been used. Biocide MIC determination used in our study has shown to be reliable in determination of CHX susceptibility.[14,24] We tested both MIC and MBC of CHX and found a correlation between them among particular bacterial species. Information about biocide MIC and MBC is crucial in clinical practice when low CHX concentrations (0.05–0.12%) are used in mucous membrane antisepsis. Despite the fact that concentrations of antiseptics used in mucous membranes are usually higher than MIC and MBC to most bacteria, the presence of mucus and dilution with body fluids may significantly lower actual concentration in vivo.

**Table III** *Correlation coefficient (P value) between MICs of different antibiotics and CHX MIC, CHX MBC of Gram-negative and Gram-positive bacteria*

| | Gram-negative bacteria | | Gram-positive bacteria | |
|---|---|---|---|---|
| Antibiotics | CHX MIC | CHX MBC | CHX MIC | CHX MBC |
| Methicillin | | | NS* | 0.735 (<0.001) |
| Ampicillin | | | NS | NS |
| Amoxicillin | | | NS | 0.612 (0.004) |
| Piperacillin | 0.672 (0.001) | 0.473 (0.023) | | |
| Piperacillin/Tazobactam | 0.555 (<0.0001) | 0.480 (0.002) | | |
| Amoxicillin/Clavulanic acid | | | NS | 0.529 (0.016) |
| Cefalothin | | | NS | 0.638 (0.002) |
| Cefuroxime | | | NS | 0.770 (<0.001) |
| Cefotaxime | 0.542 (<0.001) | 0.547 (<0.001) | | |
| Ceftazidime | 0.446 (0.004) | 0.349 (<0.001) | | |
| Cefepime | 0.538 (<0.001) | 0.513 (0.001) | | |
| Aztreonam | 0.414 (0.008) | 0.408 (0.011) | | |
| Imipenem | 0.379 (0.016) | 0.603 (<0.001) | | |
| Gentamicin | 0.551 (<0.001) | 0.396 (0.014) | 0.686 (<0.001) | 0.412 (0.024) |
| Amikacin | 0.328 (0.039) | NS | | |
| Ciprofloxacin | 0.595 (<0.001) | 0.598 (<0.001) | | |
| Erythromycin | | | NS | NS |
| Clarithromycin | | | NS | 0.756 (<0.001) |
| Clindamycin | | | NS | NS |

*NS, not significant.

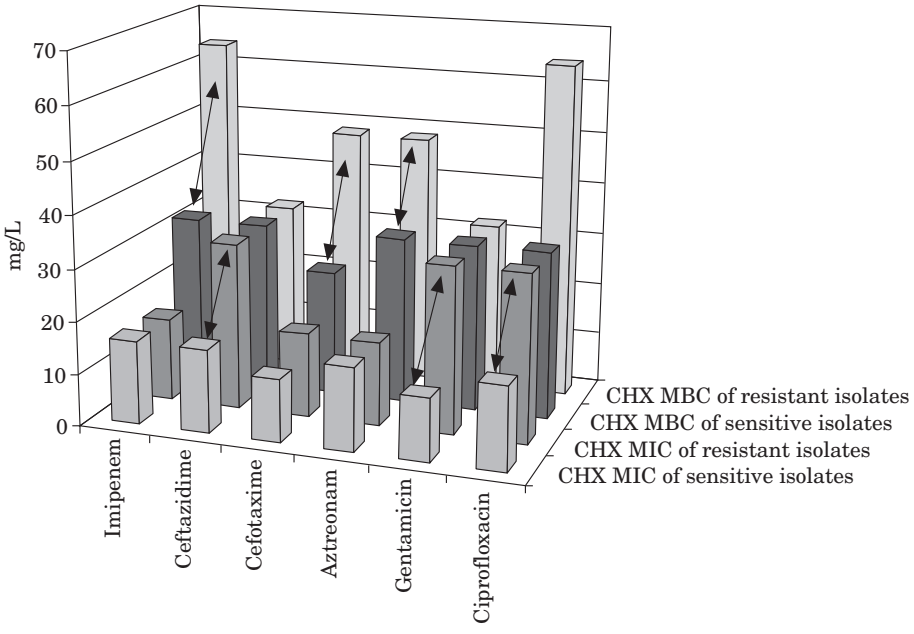


**Figure 3** Chlorhexidine susceptibility (medians of MIC, MBC; mg/L) of antibiotic-sensitive vs. antibiotic-resistant Gram-negative isolates (arrows indicate statistical differences between groups, $P < 0.05$).

Increasing frequency of hospital infection leads to overuse and pressure of biocides, similarly to antibiotics. The linkage between bacterial resistance and the use of biocides has been suggested.[24] Experiments of *Pseudomonas stutzeri* strains with induced CHX resistance have shown an increase in resistance to polymyxin B, gentamicin, nalidixic acid, erythromycin and ampicillin.[24] Increased resistance to glutaraldehyde of *Mycobacterium chelonae* was combined with increases in the MICs of rifampicin and ethambutol but not isoniazid.[27] In our study, elevated CHX MICs and MBCs of Gram-negative bacteria correlated with the MIC of different antibiotics. Non-fermentative bacteria are

often multi-resistant to antibiotics and mainly associated with hospital infections in intensive care units.[28] *A. baumannii* and *P. aeruginosa* in our study revealed high inhibitory concentrations of antibiotics and CHX.

We found cross-resistance between CHX and several antibiotics such as beta-lactams, quinolones and aminoglycosides. Combined resistance of antibiotics and biocides can be presumed by a similar kind of action of both types of agents. CHX acts through the damage of the outer cell layers and crosses the cell wall or outer membrane, then attacks the bacterial cytoplastic or inner membrane causing leakage of intracellular constituents.[1,29,30] Changes in outer or inner membranes, but also efflux pumps have been implicated in bacterial resistance to biocides and antibiotics.[24,31,32] As shown by electronmicroscopic investigations, no structural damage of the outer membrane of CHX-resistant isolates of *P. stutzeri* could be seen after CHX exposure.[33] However, CHX-sensitive isolates showed peeling of the outer membrane, a substantial loss of cytoplasmic electron-dense material and extensive lysis. Experiments with cell-wall-deficient *Providencia stuartii* revealed the possible role of the inner membrane in CHX resistance.[34]

In our study, non-fermentative bacteria showed the highest inhibitory concentrations to CHX. Similarly, CHX resistance of *P. aeruginosa* and *A. baumannii* has been detected in different countries.[35,36] On the contrary, Barry *et al.*[17] demonstrated non-fermentative bacteria to be relatively CHX sensitive. However, CHX has been demonstrated to be reliable against enterobacteria, *Neisseria gonorrhoeae* and also to intracellular microbes such as *Chlamydia trachomatis*.[25,37–39] Concerning enterobacteria, we found *K. pneumonia* to be less susceptible to CHX than *E. coli*. Also in previous studies, *E. coli* has been shown to be more sensitive to CHX than other enterobacteria.[40]

Inhibition of growth of *S. pyogenes* was achieved at the lowest CHX concentrations. The same tendency was found while studying CHX susceptibility of oral streptococci.[41] All our *S. aureus* isolates responded to CHX at low concentrations. However, in some studies, CHX has proved to be less bactericidal against methicillin-resistant than methicillin-susceptible *S. aureus*.[12,13] In our study, only methicillin-susceptible isolates were used.

Among Gram-positive bacteria, *E. faecalis* was most resistant to CHX, having an MIC comparable to the MIC of *E. coli*, whereas MBC appeared to be even higher. Kampf *et al.*[11] have defined CHX susceptibility of enterococci to be concentration and exposure time dependent in quantitative suspension tests. They found *E. faecium* to be less susceptible than *E. faecalis*.

Our finding that strains resistant to antibiotics were usually less susceptible to CHX leads to practical consequences. (1) Selective pressure due to usage of low concentrations of CHX may select strains which are more resistant to antibiotics and thus increase overall antibiotic resistance. (2) In clinical departments, such as intensive care units, where bacteria are usually more resistant due to higher antibiotic pressure, efficacy of some disinfectants should be tested. (3) Since there are no available simple methods for routine testing of susceptibility of bacteria to disinfectants, antibiotic resistance patterns could be used for biocide effect prediction.

We conclude that data about microbial species and antibiotic susceptibility of infectious agents may suggest CHX susceptibility. This information might be especially valuable concerning antibiotic-resistant hospital pathogens in the intensive care units where increased CHX concentrations may be required. Genetic linkage between bacterial resistance to antibiotics and biocides still needs to be determined.

## Acknowledgements

This study was supported by the grants from Estonian Ministry of Education (No. 0418), Estonian Science Foundation (No. 4392) and Swedish Institute of Infcctious Diseases Control (Baltic project).

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