Int J Infect. 2017 April; 4(2):e40296. doi: 10.5812/iji.40296.

Published online 2016 August 30. **Research Article**

Antibiotic Resistance Induction by Benzalkonium Chloride Exposure in Nosocomial Pathogens

Azizollah Ebrahimi,[1,*] Zohreh Arvaneh,[2] Mohamadreza Mahzounieh,[1] and Sharareh Lotfalian[1]

[1]Department of Pathobiology, School of Veterinary Science, Shahrekord University, Shahrekord, IR Iran

[2]Department of Pathobiology,Veterinary College, Shahrekord University, IR Iran

[*]*Corresponding author*: Azizollah Ebrahimi, Department of Pathobiology, School of Veterinary Science, Shahrekord University, Shahrekord, IR Iran. Tel: +98-3832324427, Fax: +98-3832324427, E-mail: A_kahrizsangi@yahoo.com

Received 2016 June 22; **Revised** 2016 August 03; **Accepted** 2016 August 03.

Abstract

Background: Biocides (disinfectants) are crucial for controlling various infections and are widely used in environments for the control of microorganisms. Exposure of bacteria to biocides can select for mutants with decreased biocide susceptibility that often display a decrease in susceptibility to antibiotics.

Objectives: The present work was done during 14 months from February 2015, at the microbiology laboratory of the Veterinary college of Shahrekord university with the aim of investigating the impact of benzalkonium chloride exposure on antibiotic resistance in some common nosocomial pathogens.

Methods: Standard strains of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli* and *Staphylococcus aureus* were used, and in parallel to each standard strain four hospital isolates collected from Shahrekord and Esfahan hospitals were examined. Tube double serial dilution method was used for determination of minimum inhibitory concentration (MIC) of antibiotics and Benzalkonium Chloride (BKC). Spontaneous mutants were developed by exposure of examined species to BKC, and their mean MICs to examined drugs were evaluated.

Results: The mean MICs of the BKC and antibiotics used in this study were not similar between the parent and mutant strains of the examined isolates. In *E. coli*, *A. baumannii* and *S. aureus* isolates, differences between the mean MICs of BZK and ciprofloxacin had a similar pattern and were statistically significant ($P < 0.05$). In isolates of *P. aeruginosa*, differences between parent and mutant isolates for all of the tested drugs were significant.

Conclusions: Vast use of BKC in various environments and their accumulation represents a potential risk for selective pressure towards selection of bacteria with decreased antibiotic susceptibility.

Keywords: *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *E. Coli*, *Staphylococcus aureus*, Benzalkonium Chloride, Antibiotics

1. Background

Gram-negative pathogenic bacteria are commonly resistant to many antimicrobial compounds by multiple mechanisms including reduced outer membrane permeability, and active efflux mechanisms by efflux pumps. Selection of antibiotic-resistant mutants that have been exposed to antibiotics can lead to distribution of drug resistance among various bacterial species (1).

Biocides (disinfectants) are crucial for controlling various infections and are widely used in environments for the control of microorganisms (2). They are active compounds that exhibit toxicity against target organisms. It seems that major mechanisms of antibiotic resistance are also involved in biocide resistance (3).

Exposure of bacteria to biocides can select for mutants with decreased biocide susceptibility that often display a decreased susceptibility to antibiotics, thus biocides may act as an agent of antibiotic resistance. Moreover, it has been reported that exposure of bacteria to biocides, at concentrations below those required to arrest growth ,can also select for antibiotic resistant strains (4, 5).

Also the vast use of biocides and their accumulation in the environment could lead to selection of biocide resistant bacteria, which could be cross resistant to antibiotics (5).

Among biocides, quaternary ammonium compounds (QACs) are the most useful antiseptics and disinfectants. Benzalkonium chloride (BKC) is a QACs demonstrating broad-spectrum antimicrobial activity. Low level and greater magnitude of adaptation to biocide has been noted in strains of *Pseudomonas aeruginosa* (6). Some studies demonstrated a relationship between increased *Staphylococcus aureus* MICs to the β-lactam oxacillin and biocide (3). A report by Carla et al. indicated that in *S. aureus*, the presence of qacA/B gene confers higher resistance to benzalkonium chloride and suggested that factors associated with methicillin resistant phenotype may confer resistance to BKC, even though tolerance appeared to be higher for me-

thicillin sensitive isolates (7).

Bore et al. reported that *E. coli* K-12 adapted to higher tolerance for BKC, acquired several general resistance mechanisms related to multiple antibiotic resistance (8). In a recent report published by Vijaya et al., it was indicated that 22% of multi drug resistant *Pseudomonas aeroginosa* isolates showed reduced susceptibility to BKC (9).

Significant positive correlations were also reported by Kawamura Sato et al., who studied the MICs of different biocides including BKC, for 283 clinical Acinetobacter isolates (10). In addition to induction of antibiotic resistance, biofilm formation induction was also observed for pathogens in the presence of BKC sub MIC (11).

2. Objectives

The aim of this study was to examine the exposure effects of major nosocomial pathogens to BKC, as a commonly used disinfectants.

3. Methods

3.1. Bacterial Cultures and Materials

The study was done during 14 months, from February 2015 to April 2016, at the microbiology laboratory of the veterinary college of Shahrekord university. The present work started with collection of hospital isolates of the studied microorganisms and their preliminary analysis for final confirmation.

The studied microorganisms included the reference strains of *Pseudomonas aeruginosa* ATCC 9027 and *Acinetobacter baumannii* NCTC 13305, kindly provided by Dr B. Zamanzad and Dr A. Gholipour (Dept. of microbiology, Shahrekord Medical school), *E. Coli* ATCC 25922 and *S. aureus* RTCC 2465 kindly provided by Dr H. Motamedi (Dept. of microbiology, College of basic Science, Shahid Chamran University). In addition, in parallel to each standard strain, four hospital isolates from Shahrekord and Esfahan hospitals were used. Early morphological and biochemical tests were done to confirm the genera and species of the received isolates. The methods for isolation and identification of all isolates were based on the guidelines of James et al. (12).

The strains were kept in Lauria Bertani broth (LB broth) at 4°C and sub-cultured on appropriate agar plates 24 hours prior to examinations. Mueller Hinton Broth (MHB) was used for all the antibacterial assays. Ciprofloxacin (CAS Number 85721-33-1), tetracycline (CAS Number 60-54-8), penicillin (CAS Number 69-57-8) and kanamycin (CAS Number 25389-94-0) (all in powder form and from Sigma-Aldrich), as fluoroquinolone, tetracyclines, penicillins and aminoglycoside representative, respectively, were used. Solid (Light Yellow) BKC was purchased (Sigma, CAS Number: 63449-41-2) and a dilution of one percent solution was prepared and preserved for further use.

3.2. Antimicrobial Testing

Aqueous form of the drugs was prepared using solvents and recommendations of the producer company. Tube double serial dilution method was used for determination of Minimum Inhibitory Concentration (MIC) of antibiotics and BKC against four standard and 16 hospital isolates, according to guidelines of the Clinical and Laboratory Standards Institute (13). Briefly, series of Muller Hinton broth (MHb) containing tubes of different concentrations of drugs were designed. The bacterial cultures were incubated aerobically at 37°C for 18 to 24. The turbidity of the cultures was adjusted to 0.5 McFarland (1.5×10^8 CFU/mL) and then diluted in saline solution, to obtain an inoculum of 5×10^6 CFU/ tube.

The two last tubes were considered as positive and negative controls. The inoculated tubes were aerobically incubated for about 18 hours at 37°C. The lowest concentration that inhibited visible growth after incubation was defined as MIC. All assays were performed in triplicates.

3.3. Mutant Development

Spontaneous mutants developed by modification of the method of Ricci et al. (14). Briefly, spontaneous mutants for each isolate were developed by culturing the parent strains in tubes of MHb, containing four MICs of BKC. Tubes were incubated at 37°C for 24 - 48 hours, followed by centrifugation for 10 minutes at 1600 rpm. Samples from sediment of each tube were sub-cultured on Nutrient Agar (NA) plates containing four MICs of BKC and incubated as above.

One colony with the typical size and morphology of the original strain was chosen randomly from each of the latter NA plates and sub-cultured onto BKC free Tryptic Soy Broth (TSB) and incubated at 37°C for 24 hours. These spontaneous mutants were examined for determination of their MICs for antibiotics by the above-mentioned method (14).

Statistical analysis and interpretation of data was performed using the Chi square test.

4. Results

In the parent strains of *E. coli*, *S. aureus*, *P. aeruginosa* and *A. baumannii* isolates, the mean MICs of BKC varied between 5, 10, 24 and 26.66 ug/mL, respectively (Table 1).

The most resistant species to ciprofloxacin and penicillin was the parent strains of *P. aeruginosa* while, for tetracycline and kanamycin this was the parent strains of *A. baumannii* (Table 1). In mutant strains similar respective pattern of resistance to antibiotics was observed.

The mean MICs of the BKC and antibiotics are not similar between the parent and mutant strains of the examined isolates.

Differences between the mean MICs of penicillin between parent and mutants of all species are statistically significant ($P < 0.05$), (Table 1).

For *E. coli*, *A. baumannii* and *S. aureus* isolates, differences between the mean MICs of BZK and ciprofloxacin had a similar pattern and were statistically significant ($P < 0.05$), (Table 1). In isolates of *P. aeruginosa*, differences between parent and mutant isolates for all of the tested drugs were significant ($P < 0.05$), (Table 1).

5. Discussion

Benzalkonium chloride is a QAC, with broad-spectrum antimicrobial activity. Its vast use could lead to accumulation in the environment and selection of biocide resistant bacteria, which could be cross-resistant to antibiotics (4). In the current work we showed that exposure of the examined nosocomial bacterial pathogens to BKC resulted in the selection of mutants with decreased susceptibilities (Table 1) to examined antibiotics. Although in some cases (tetracycline and kanamycin for *E. coli*, *A. baumannii* and *S. aureus*) differences in mean MICs of parent and mutant strains were not statistically significant.

In our mutant strains of *S. aureus*, only susceptibility to penicillin was significantly less than the parent strains ($P < 0.05$).

Several staphylococcal clinical isolates resistant to BKC have been checked for antibiotic susceptibilities. A genetic linkage was reported between resistance to BKC products and penicillin (15). Our results are in agreement with Carla et al., who reported that in *S. aureus* the presence of qacA/B gene confers higher resistance to benzalkonium chloride and suggested that factors associated with methicillin resistant phenotype may confer resistance to BKC (7).

In *P. aeroginosa* mutant strains, significant decrease in susceptibilities to all examined drugs was observed. The effect of BKC on induction of antimicrobial resistance in *P. aeroginosa* was investigated by Tandukar et al. (2013), who reported that increased resistance to BKC led to decreased susceptibility to penicillin G, tetracycline and ciprofloxacin. They also demonstrated that increased resistance to BKC and penicillin G involves degradation or transformation of BKC, whereas resistance to tetracycline and ciprofloxacin is due to the activity of efflux pumps (16). In a recent report in this regard Vijaya et al. indicated that 22% of multi drug resistant *P. aeroginosa* isolates showed reduced susceptibility to BKC (9).

As indicated by Table 1, mutant strains of *A. baumannii* showed a significant decrease in susceptibilities to penicillin and ciprofloxacin. These observations are in agreement with the report of Fernandez-Cuenca et al. (2015), indicating reduced susceptibility to BKC, which was associated with resistance to aminoglycosides, tetracycline and ciprofloxacin (17). Significant positive correlations were also reported by Kawamura Sato et al., who tried to determine the MICs of different biocides including BKC for 283 clinical Acinetobacter isolates (10).

Our data showed ($P < 0.05$), (Table 1) a significant decrease in susceptibilities of mutant strains of *E. coli* to tetracyclin, penicillin and ciprofloxacin. Our results are in line with Bore et al.'s report, indicating that *E. coli* K-12 adapted to higher tolerance to BKC, had acquired several general resistance mechanisms related to multiple antibiotic resistance (8). In a recent published report, it was demonstrated that there is a cross resistance between BKC and glutaraldehyde-based disinfectants towards tetracycline, ciprofloxacine, ampicillin and other antimicrobials (18).

Irrespective of the induction of antibiotic resistance by BKC in our examined species, our earlier work indicated that BKC, at sub MIC concentrations, could enhance biofilm formation by pathogenic bacteria (11).

Vast use of biocides in various environments and their accumulation represents a potential risk for selective pressure towards selection of bacteria with decreased biocide susceptibility, which could be cross resistant to various antibiotics (19). Further investigation to evaluate such selective pressure in hospitals, farms and health care environments is suggested.

Acknowledgments

The authors thank Dr S. Habibian (Dept. of pharmacology, Veterinary college, Shahrekord University) for his kind corporation in preparation of the examined antibiotics, and Dr B. Zamanzad, Dr A. Gholipour (Dept. of microbiology, Shahrekord medical school) and Dr H. Motamedi (Dept. of microbiology, college of basic Science, Shahid Chamran University) for their kind corporation in preparing the examined standard strains.

Footnotes

Authors' Contribution: Azizollah Ebrahimi, designed and supervised the study and wrote the manuscript; Zohreh Arvaneh, followed the examinations and arranged

Table 1. Mean of Minimum Inhibitory Concentration (MIC), (ug/mL.) of Benzalkonium Chloride and Antibiotics for Parent and Mutant Strains of *E. coli*, *A. baumannii*, *S. aureus* and *P. aeruginosa*

	Bacteria			
	E. coli	***A. baumannii***	***S. aureus***	***Ps. aeruginosa***
Parent means MICs				
BKC	5	26.66	10	24
Tet.	3.33	1055	3.5	209.2
Pen.	825	1041.67	706.2	10000
Kan.	22.87	286	56.82	24.96
Cip.	0.42	0.5	0.31	56.2
Mutant means MICs				
BKC	10^{S}	193.33^{S}	40^{S}	96^{S}
Tet.	6.66^{N}	2110^{N}	7^{N}	3497.6^{S}
Pen.	1025^{S}	40083.33^{S}	1412.44^{S}	98750^{S}
Kan.	45.74^{N}	572^{N}	113.64^{N}	112.48^{S}
Cip.	$.825^{S}$	2^{S}	$.625^{N}$	62.4^{S}

Abbreviations: BKC, Benzalkonium chlorid; Cip, Ciprofloxacin; N, differences between mutant and parent strains are not significant; Kan, Kanamycin; Pen, Penicillin; S, differences between mutant and parent strains are significant; Tet, Tetracyclin.

the data sheet; Mohamadreza Mahzounieh, supervised the study; Sharareh Lotfalian, provided technical supports.

Funding/Support: This work was supported by the Veterinary college of Shahrekord university, and was part of an MSC thesis research project.

References

1. Piddock LJ. Mechanisms of fluoroquinolone resistance: an update 1994-1998. *Drugs.* 1999;**58 Suppl 2**:11–8. [PubMed: 10553699].
2. Fraise AP. Choosing disinfectants. *J Hosp Infect.* 1999;**43**(4):255–64. [PubMed: 10658801].
3. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev.* 1999;**12**(1):147–79. [PubMed: 9880479].
4. Sheldon AJ. Antiseptic "resistance": real or perceived threat?. *Clin Infect Dis.* 2005;**40**(11):1650–6. doi: 10.1086/430063. [PubMed: 15889364].
5. Webber MA, Whitehead RN, Mount M, Loman NJ, Pallen MJ, Piddock LJ. Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. *J Antimicrob Chemother.* 2015;**70**(8):2241–8. doi: 10.1093/jac/dkv109. [PubMed: 25953808].
6. Langsrud S, Sundheim G, Borgmann-Strahsen R. Intrinsic and acquired resistance to quaternary ammonium compounds in food-related Pseudomonas spp. *J Appl Microbiol.* 2003;**95**(4):874–82. [PubMed: 12969304].
7. Raggi C, Filippini P, Monaco M, Pantosti A, Creti R, Baldassarri L. Methicillin resistance, biofilm formation and resistance to benzalkonium chloride in Staphylococcus aureus clinical isolates. *Clin Microbiol.* 2013.
8. Bore E, Hebraud M, Chafsey I, Chambon C, Skjaeret C, Moen B, et al. Adapted tolerance to benzalkonium chloride in Escherichia coli K-12 studied by transcriptome and proteome analyses. *Microbiology.* 2007;**153**(Pt 4):935–46. doi: 10.1099/mic.0.29288-0. [PubMed: 17379704].
9. Vijaya K, Aboody MS, Alfanaisan MK, Sandle T. In vitro susceptibility of multidrug resistant pseudomonas aeruginosa clinical isolates to common biocides. *Int J Res Pharm Sci.* 2016;**7**(1):110–6.
10. Kawamura-Sato K, Wachino J, Kondo T, Ito H, Arakawa Y. Correlation between reduced susceptibility to disinfectants and multidrug resistance among clinical isolates of Acinetobacter species. *J Antimicrob Chemother.* 2010;**65**(9):1975–83. doi: 10.1093/jac/dkq227. [PubMed: 20576639].
11. Ebrahimi A, Hemati M, Shabanpour Z, Dehkordi SH, Bahadoran S, Lotfalian S, et al. Effects of benzalkonium chloride on planktonic growth and biofilm formation by animal bacterial pathogens. *Jundishapur J Microbiol.* 2015;**8**(2).
12. James HJ, Michael AP. Manual of Clinical Microbiology. 11 ed. Washington, DC: ASM Press; 2015.
13. Wayne P. Clinical and Laboratory Standarts Institute: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that grow Aerobically. USA: Approved Standart; 2006.
14. Ricci V, Tzakas P, Buckley A, Piddock LJ. Ciprofloxacin-resistant Salmonella enterica serovar Typhimurium strains are difficult to select in the absence of AcrB and TolC. *Antimicrob Agents Chemother.* 2006;**50**(1):38–42. doi: 10.1128/AAC.50.1.38-42.2006. [PubMed: 16377664].
15. Sidhu MS, Heir E, Leegaard T, Wiger K, Holck A. Frequency of disinfectant resistance genes and genetic linkage with beta-lactamase transposon Tn552 among clinical staphylococci. *Antimicrob Agents Chemother.* 2002;**46**(9):2797–803. [PubMed: 12183231].
16. Tandukar M, Oh S, Tezel U, Konstantinidis KT, Pavlostathis SG. Long-term exposure to benzalkonium chloride disinfectants results in change of microbial community structure and increased antimicrobial resistance. *Environ Sci Technol.* 2013;**47**(17):9730–8. doi: 10.1021/es401507k. [PubMed: 23924280].
17. Fernandez-Cuenca F, Tomas M, Caballero-Moyano FJ, Bou G, Martinez-Martinez L, Vila J, et al. Reduced susceptibility to biocides in Acinetobacter baumannii: association with resistance to antimicrobials, epidemiological behaviour, biological cost and effect on the expression

of genes encoding porins and efflux pumps. *J Antimicrob Chemother.* 2015;**70**(12):3222–9. doi: 10.1093/jac/dkv262. [PubMed: 26517560].
18. Nhung NT, Thuy CT, Trung NV, Campbell J, Baker S, Thwaites G, et al. Induction of Antimicrobial Resistance in Escherichia coli and Non-Typhoidal Salmonella Strains after Adaptation to Disinfectant Commonly Used on Farms in Vietnam. *Antibiotics (Basel).* 2015;**4**(4):480–94. doi: 10.3390/antibiotics4040480. [PubMed: 27025637].
19. Mathews S, Henderson S, Reinhold D. Uptake and accumulation of antimicrobials, triclocarban and triclosan, by food crops in a hydroponic system. *Environ Sci Pollut Res Int.* 2014;**21**(9):6025–33. doi: 10.1007/s11356-013-2474-3. [PubMed: 24464075].