

Original Article



Reduced Susceptibility to Biocides among Enterococci from Clinical and Non-Clinical Sources

Mohammad Hossein Sobhanipoor ¹, Roya Ahmadrajabi ¹,
Hossein Hosseini Nave ¹, and Fereshteh Saffari ²

¹Department of Medical Microbiology, Afzalipoor Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Iran

²Medical Mycology and Bacteriology Research Center, Kerman University of Medical Sciences, Kerman, Iran

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Corresponding Author:





Fereshteh Saffari, PhD

Department of Microbiology and Virology,
Kerman University of Medical Sciences, 22
Bahman Blvd, Kerman, Iran.
Tel/Fax: +98-3433257665
E-mail: fsaffari@kmu.ac.ir

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ORCID iDs

Mohammad Hossein Sobhanipoor 
<https://orcid.org/0000-0002-7670-4325>
Roya Ahmadrajabi 
<https://orcid.org/0000-0001-5636-7037>
Hossein Hosseini Nave 
<https://orcid.org/0000-0002-9152-818X>
Fereshteh Saffari 
<https://orcid.org/0000-0001-7281-7077>

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Conflict of Interest

No conflicts of interest.

ABSTRACT

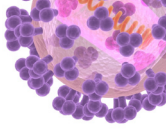
Background: Wide use of biocidal agents such as benzalkonium chloride (BCC) and chlorhexidine digluconate (CHX) in hospitals and non-hospital environments, has raised concerns over the emergence of non-susceptible strains. Efflux pumps are of known main mechanisms in biocide tolerance which have been rarely addressed in enterococci - members of gut microbiota which can cause serious problems particularly in hospitalized patients. The purpose of this study was to investigate the susceptibility of enterococci from different sources (clinical and fecal isolates) toward BCC and CHX, and its correlation with efflux associated genes. Also, possible link between biocide tolerance and antibiotic resistance was examined.

Materials and Methods: One hundred and four enterococcus isolates including clinical (n = 54) and fecal isolates (n = 50) were studied for susceptibility toward BCC, CHX, ciprofloxacin, gentamicin and vancomycin. Twelve efflux associated genes were investigated by polymerase chain reaction assay.

Results: In clinical isolates, reduced susceptibility to CHX and resistance to gentamicin and ciprofloxacin were significantly higher than fecal isolates. Vancomycin resistance was associated with increasing minimum inhibitory concentration of CHX. Among all investigated genes, only three ones, *efrA*, *efrB* and *emeA* were detected which were significantly associated with reduced susceptibility to CHX and were more frequent among clinical isolates. Also, high level resistance to gentamicin was significantly associated with the presence of *efrA/B* as well as with reduced susceptibility to CHX.

Conclusion: As expected, reduced susceptibility to CHX, was significantly higher in clinical isolates. However, the presence of a vancomycin-resistant enterococci among fecal isolates of healthy people which showed resistance/tolerance to studied antimicrobial agents, was unexpected and highlights the need to investigate other non-hospital environments to avoid dissemination of antimicrobial resistance. Correlation between reduced susceptibility to CHX and high level resistance to gentamicin, substantiates monitoring of biocide tolerance particularly in the healthcare settings to control the establishment of antimicrobial resistant strains.

Keywords: Biocide; Efflux; Enterococcus; Healthcare environment; Antibiotic resistance



Author Contributions

Conceptualization: FS, RA, HHN. Data curation: MHS. Formal analysis: FS, RA, MHS. Investigation: MHS. Writing - original draft: FS, MHS. Writing - review & editing: FS, MHS, RA, HHN.

INTRODUCTION

Enterococcus spp. are organisms that can be isolated from both hospital and non-hospital environments. They are members of gut microbiota and can be used as general markers for fecal pollutions. Furthermore, despite not having many virulence factors, they can cause serious problems particularly in hospitalized patients [1–3].

Biocidal agents such as benzalkonium chloride (BCC) and chlorhexidine digluconate (CHX) are widely used in hospitals and healthcare facilities, as well as household settings, farms and food industries. BCC is a quaternary ammonium compound (QAC) which can denature cytoplasmic membrane proteins [4–6]. CHX is a water-soluble biguanide which by protein denaturation and inhibition of enzymes in the membranes, promote membrane permeability and inhibit cell growth [6–8].

Reduced susceptibility to biocides can be conferred by intrinsic mechanisms and active efflux pumps which the latter is frequently recognized as the main mechanism of biocide tolerance [9]. Multidrug efflux pumps can actively force out a broad range of compounds (may be structurally unrelated) including biocides, dyes and antibiotics, leading to development of multi-resistant strains [10]. In enterococci, there are some known multidrug efflux pumps from major facilitator superfamily (MFS), including EmeA, QacA and QacB which contribute in resistance to aminoglycosides, fluoroquinolones and reduced BCC susceptibility. The others are QacE, QacF, QacG, QacZ and Smr which belong to small multidrug resistance (SMR) family, and EfrA, EfrB as ATP-binding cassette family efflux pumps [4, 11, 12].

In most previous studies, the presence of pathogenic bacteria with reduced susceptibility to commonly used biocides in the healthcare settings, have been the focus of attention [4, 13]. However, wide use of biocides in areas other than healthcare environments have recently raised concerns over the emergence of non-susceptible strains in the non-healthcare environments. This issue becomes even more important when the development of simultaneous resistance to biocides and antibiotics is proposed [14]. This may be the result of cross- or co-resistance between antibiotics and biocides, or even may be caused by the clonal spread of resistant strains. Anyway, there are inconsistencies in the reports on a link between antibiotic resistance and reduced susceptibility to biocides. The present study aimed to evaluate the susceptibility of enterococci from different sources (clinical and fecal isolates) to two antiseptic agents, BCC and CHX, and to examine if there is any relationship between non-susceptibility to biocides and resistance to commonly used antibiotics (ciprofloxacin, gentamicin). In addition, efflux genes implicated in this issue were investigated.

MATERIALS AND METHODS

1. Bacterial isolates

Totally, 104 enterococcus isolates from bacterial collection in microbiology department of Kerman University of Medical Sciences were included in this study. They were clinical isolates (n = 54) from different specimens including urine (n = 51), blood (n = 1), cerebrospinal fluid (n=1) and wound (n = 1), and non-clinical isolates (n = 50) collected from feces of healthy volunteers. All isolates had been identified to the species level using biochemical and molecular tests.

2. Statement of Ethics

The present study was approved by the Ethics Committee of the Research Council of Kerman University of Medical Sciences, Kerman, Iran (IR.KMU.REC.1398.706).

3. Susceptibility tests

1) Disinfectants

Minimum inhibitory concentrations (MICs) for chlorhexidine (Merck, Feltham, United Kingdom) and benzalkonium chloride (ACROS Organics, Geel, Belgium) were determined using 0.25 - 128 µg/ml concentration ranges (two fold dilution) by broth microdilution method. The lowest concentration of disinfectant that inhibited the growth of bacteria, was considered as MIC. *Enterococcus faecalis* American type culture collection (ATCC) 29212 was used as control. In this study, respectively, MIC ≥ 64 µg/ml and MIC ≥ 8 µg/ml for CHX and BCC, were considered as reduced susceptibility to related agents [15, 16].

2) Antibiotics

At first, using disk diffusion method, the susceptibility pattern of isolates was determined against gentamicin (120 µg), ciprofloxacin (5 µg) and vancomycin (30 µg) (Liofilchem Co, Roseto degli Abruzzi, Italy). Then, based on the results, the following concentration ranges were used for detection of MIC by broth microdilution method as recommended by clinical and laboratory standards institute [17]: gentamicin (15.625 – 8,000 µg/ml) and ciprofloxacin (1 – 512 µg/ml) (Exir pharmaceutical company, Borujerd, Lorestan, Iran).

3) Detection of efflux pump genes

All isolates were screened for the presence of genes encoding efflux pumps using polymerase chain reaction (PCR). PCR experiments were carried out using primers specific for *qacA/B*, *qacC*, *qacG*, *qacJ*, *qacZ*, *qacΔE*, *oqxA*, *oqxB*, *emeA*, *efrA*, *efrB* and *smr* genes as described previously [4, 18, 19] (Table 1).

4. Statistical analysis

The distribution genes encoding efflux pumps in clinical and non-clinical isolates was analyzed using the Chi-square and Fisher's exact tests for each gene by SPSS (IBM Corp., Armonk, NY, USA). $P \leq 0.05$ was considered statistically significant.

RESULTS

The MICs of CHX in clinical and non-clinical isolates ranged from 16 to 128 µg/ml and 8 to 128 µg/ml, respectively. In this case, 38% (n = 19) of non-clinical and 63% (n = 34) of clinical isolates showed reduced susceptibility to CHX (MIC ≥ 64 µg/ml).

Regarding BCC, MICs level were 2 - 8 µg/ml and 2-16 µg/ml in clinical and non-clinical isolates, respectively. According to the results, reduced susceptibility to BCC (MIC ≥ 8 µg/ml) was significantly higher in non-clinical isolates, 52% (n = 26) vs. clinical isolates 28% (n = 15) ($P = 0.01$).

Antibiotic susceptibility testing showed that respectively, 52% (n = 26) and 70% (n = 38) of non-clinical and clinical isolates, were resistant to ciprofloxacin and these values were 18% (n = 9) and 74% (n = 40) for gentamicin [known as high level gentamicin resistant (HLGR)]. Totally, 18% (n = 19) of isolates (one isolate from non-clinical group), were detected as

Table 1. The oligonucleotide primers used for amplification of genes encoding efflux pumps

Target gene Primer	Primer Sequence (5-3)	Amplicon size (bp)
<i>qacZ</i>	F-ATATTGCTTACGCAAGTTGG R-AGTGTGATGATCCGAATGTG	156
<i>qacG</i>	F-CAACAGAAATAATCGGAACT R-TACATTTAAGAGCACTACA	275
<i>qacJ</i>	F-GGCCAACATTAGGCACACTTA R-TGACTTGATCCAAAAACGTTAAGA	232
<i>qacΔE</i>	F-GGCTTTACTAAGCTTGCCCC R-AGCCCCATACCTACAAAGCC	127
<i>oqxA</i>	F-GGGGAAACATTACCTGAC R-ACGGGAGACGAGGTTGGTA	407
<i>oqxB</i>	F-CTCGCTCGTCTTATGTCAAT R-TTACAGGCCAGGCCATC	567
<i>emeA</i>	F-AGCCCAAGCGAAAAGCGTTT R-CCATCGCTTTCGGACGTTCA	128
<i>efrA</i>	F-TTGGCTTTATGACGCCAGT R-ATGCGCGTATTACCCGCAA	224
<i>efrB</i>	F-TAGTGATGATGTTCTTAATCAA R-ATTGACTTGTTAAAGCCTTCA	211
<i>qacA/B</i>	F-GCAGAAAGTGCAGAGTTTCG R-CCAGTCCAATCATGCCTG	361
<i>qacC</i>	F-AAACAATGCAACACCTACCACT R-AACGAAACTACGCCGACTATG	157
<i>smr (qacC + qacD)</i>	F-GCCATAAGTACTGAAGTTATTGGA R-GACTACGGTTGTTAAGACTAAACCT	195

vancomycin resistant enterococci (VRE). Interestingly, VRE isolate from fecal specimen, showed resistance to tested antibiotics as well as reduced susceptibility to CHX and BCC.

In this study, the MIC₉₀ and MIC₅₀ of CHX (128 µg/ml and 64 µg/ml) in VRE isolates were higher than vancomycin susceptible enterococci (VSE) (64 µg/ml and 32 µg/ml). It means that vancomycin resistance in clinical isolates was associated with increasing MIC for CHX. While, the MIC₉₀ and MIC₅₀ of BCC (8 µg/ml and 4 µg/ml) were similar in VRE and VSE isolates.

According to results, the resistance rate to ciprofloxacin, gentamicin and vancomycin, and reduced susceptibility to CHX, were significantly higher in clinical isolates in comparison to non-clinical ones ($P = 0.01$). Regarding BCC, no association was found between resistance to tested antibiotics and reduced susceptibility to BCC. However, high level gentamicin resistance was significantly associated with reduced CHX susceptibility ($P = 0.03$).

Among investigated genes encoding efflux pumps in this study, only three ones, *efrA*, *efrB* and *emeA*, were detected which were significantly ($P < 0.05$) more frequent among clinical isolates (Table 2). Furthermore, the presence of these genes was associated significantly ($P = 0.00$) with reduced susceptibility to CHX but not to BCC (Table 3). These genes were detected in all HLGR isolates and significant association was found between the presence of *efrA/B* and high level resistance to gentamicin. Conversely, there was no association between resistance to ciprofloxacin and the presence of efflux genes.

DISCUSSION

E. faecalis and *E. faecium* are the most prevalent enterococcal healthcare associated infections. They can colonize external surface of catheters and are common cause of catheter-associated

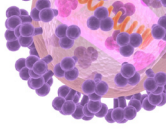


Table 2. Distribution of efflux genes among clinical and non-clinical enterococcus isolates according to antimicrobial resistance pattern

Number of isolates	Antibiotic resistance	Biocide reduced susceptibility	Efflux pump genes
Clinical			
11	CIP, GEN	CHX	<i>efrA, efrB, emeA</i>
1	CIP, GEN	CHX	<i>efrA, efrB</i>
3	-	CHX	<i>efrA, efrB, emeA</i>
1	-	CHX	<i>efrA, efrB</i>
2	GEN	-	<i>efrA, efrB, emeA</i>
1	GEN	-	<i>efrA, efrB</i>
3	CIP, GEN	BCC	<i>efrA, efrB, emeA</i>
1	CIP	-	<i>efrA, efrB, emeA</i>
2	VAN, CIP, GEN	CHX, BCC	<i>efrA, efrB, emeA</i>
2	VAN, CIP, GEN	CHX, BCC	-
1	VAN, CIP	-	<i>efrA, efrB, emeA</i>
1	VAN, CIP	CHX, BCC	-
8	VAN, CIP, GEN	-	-
1	VAN, CIP, GEN	BCC	<i>efrA, efrB</i>
1	CIP, GEN	-	<i>efrA, efrB, emeA</i>
1	GM	CHX	-
1	GM	CHX	<i>efrA, efrB, emeA</i>
1	VAN, GEN	CHX	<i>efrA, efrB, emeA</i>
1	GM	CHX, BCC	<i>efrA, efrB, emeA</i>
1	CIP	CHX	<i>efrA, efrB, emeA</i>
1	CIP	CHX	-
1	CIP, GEN	CHX, BCC	-
1	CIP, GEN	CHX	-
1	CIP, GEN	-	-
1	-	CHX, BCC	<i>efrA, efrB</i>
3	VAN	CHX	<i>efrA, efrB, emeA</i>
2	CIP, GEN	BCC	-
Non-clinical			
2	CIP, GEN	CHX	<i>efrA, efrB</i>
5	-	CHX	<i>efrA, efrB, emeA</i>
2	GEN	CHX	<i>efrA, efrB, emeA</i>
2	CIP, GEN	CHX	<i>efrA, efrB, emeA</i>
1	-	CHX	<i>efrA, efrB</i>
1	CIP	CHX, BCC	<i>efrA, efrB</i>
2	CIP	-	<i>efrA, efrB, emeA</i>
1	GEN	BCC	<i>efrA, efrB, emeA</i>
2	-	BCC	<i>efrA, efrB, emeA</i>
1	-	CHX, BCC	<i>efrA, efrB, emeA</i>
1	-	-	<i>efrA, efrB, emeA</i>
5	-	BCC	-
13	CIP	BCC	-
1	VAN, CIP, GEN	CHX, BCC	-
1	-	CHX, BCC	-
2	CIP	CHX	-
1	-	CHX	-
2	CIP	-	-
1	CIP, GEN	CHX, BCC	-
4	-	-	-

CIP, ciprofloxacin; GEN, gentamicin; CHX, chlorhexidine gluconate; VAN, vancomycin; BCC, benzalkonium chloride.

urinary tract infections. So, sanitation is important and neglecting it may lead to failure treatment [20]. On the other hand, extensive use of biocidal agents to ensure hygiene, has been lead to reduction in antiseptic susceptibility in bacteria. Although not proven, it may favor the survival of antibiotic resistant strains and consequently facilitate the transfer of resistance associated genes. For this reason, it is important that bacterial tolerance to biocides, particularly in enterococci, being monitored.

Table 3. Distribution of efflux genes among clinical and non-clinical resistant enterococcus isolates

Antimicrobial agents	Non-clinical isolates% (N)				Clinical isolates% (N)			
	CIP (15), GEN (9), VAN (1), CHX (19), BCC (26)				CIP (38), GEN (40), VAN (18), CHX (34), BCC (16)			
	<i>efrAB</i>	<i>P</i> -value	<i>emeA</i>	<i>P</i> -value	<i>efrAB</i>	<i>P</i> -value	<i>emeA</i>	<i>P</i> -value
CHX	74 (14)	0	53 (10)	0.01	82 (28)	0.01	68 (23)	0.04
BCC	19 (5)	-	15 (4)	-	53 (8)	0.1	40 (6)	-
CIP	27 (7)	-	15 (4)	-	61 (23)	0.01	53 (20)	0.2
GEN	78 (7)	0.01	56 (5)	0.1	65 (26)	0.13	55 (22)	0.3

CIP, ciprofloxacin; GEN, gentamicin; VAN, vancomycin; CHX, chlorhexidine gluconate; BCC, benzalkonium chloride.

Furthermore, little is known about biocide susceptibility profile of enterococci from non-hospital sources including fecal isolates which one would not expect to find any non-susceptibility. So, this study aimed to investigate biocide susceptibility among isolates from clinical and non-clinical sources and to examine the possibility of a link between antibiotic resistance, reduced biocide susceptibility and efflux genes.

BCC and CHX, known as time and concentration- dependent biocides, are more effective against gram positive bacteria [21, 22]. According to results, MIC of CHX in clinical isolates (16 – 128 µg/ml) was higher than reports from Turkey and Spain (6 - 64 µg/ml) and even higher than MIC against hospital acquired staphylococcal isolates from Iran (0.5 - 64 µg/ml) [15, 16, 23]. This may indicate the more exposure to CHX in our region.

Reduced susceptibility to BCC (MIC >8 µg/ml) in 28% of clinical isolates in this study, was in accordance with reports from United State of America (34%) but was lower than a report from Turkey (73%) [4, 24]. Finding BCC tolerance more frequently among non-clinical isolates (52%) can be related to multiple application of this biocide in the non-health care settings.

Comparison between reduced biocide susceptibility and vancomycin resistance showed that MIC of CHX was higher in VRE isolates similar to report of Ignak et al. [4]. In regard to BCC, no difference was found between two groups which is in accordance with Suller et al. [25] but in disagreement with Ignak et al. who again found higher MIC of BCC against VRE isolates [4]. Also, in a study from Denmark, statistically significant difference was seen in the susceptibility to BCC and CHX between VRE *faecium* and VSE *faecium* isolates [16]. Taken together, our results show that CHX tolerance can be involved in the establishment of VRE isolates in the hospital settings and emphasizes on the use of proper disinfectants in these environments. Also, the presence of one VRE isolate with high level resistance to gentamicin and ciprofloxacin and reduced susceptibility to CHX and BCC in fecal specimens, is noteworthy and substantiate the need to study more isolates from other non-hospital environments.

Contribution of efflux pumps in antiseptic resistance has been mostly reported in staphylococci [4], but some of the efflux associated genes such as *qac*, *smr* and *oqx* have been also detected in enterococci [26]. In our study, similar to Ignak et al. except for *emeA* and *efrA/B*, no other genes were found [4]. Although, the presence of these genes was significantly related to tolerance to CHX, only *efrA/B* showed significant association with high level resistance to gentamicin. This is in agreement with Lerma et al. who indicated that the expression of *efrA/B* genes increases in exposure to gentamicin [18]. Conversely, no association was found between BCC tolerance and the presence of efflux genes. Here in, role of other genes including the stress response gene *sigV* that encodes one of the extra-cytoplasmic sigma factors [27], and *gsp65* that encodes an organic hydroperoxide resistance

protein [28], overexpression of other efflux pumps, membrane composition and biofilm formation that confer tolerance to biocides, can be proposed [29].

Little is known about the relationship between reduced susceptibility to biocides and antibiotic resistance. Such a link has been demonstrated in some bacterial species including methicillin resistant *Staphylococcus aureus* [26]. Conversely, some studies have not found any evidences to substantiate such claim [26]. According to our results, high level gentamicin resistance was significantly associated with reduced CHX susceptibility ($P = 0.05$), which may indicate that the exit of both agents is done through the common mechanism or it can be assumed that over expression of efflux pumps in HLGR isolates may lead to efflux of biocides. In addition, horizontal spread of resistance genes as well as spread via clonal lineages are of the other proposed mechanisms in development of cross/ co- resistance in enterococci [26]. Similar to this finding, the increase to gentamicin resistance up to 512 fold, has been reported in *S. aureus* strains after exposure of to various sub lethal concentrations of CHX [30]. Also, in another study, it was found that after exposure of *Pseudomonas aeruginosa* to CHX, changes in the expression of efflux pump genes occur that result in gentamicin resistance [31]. Since this study was conducted on clinical isolates with different resistance mechanisms even more effective than those mediated by efflux pumps, role of these pumps in development of resistance to different antimicrobial agents may be masked and this may explain why such association was not detected for ciprofloxacin resistance.

In conclusion, as expected, resistance to gentamicin and ciprofloxacin and reduced susceptibility to CHX, was significantly higher in clinical isolates. However, the presence of a VRE isolate showing resistance/tolerance to studied antimicrobial agents among fecal isolates of healthy people, was unexpected and highlights the need to investigate other non-hospital environments to avoid dissemination of antimicrobial resistance. In addition vancomycin resistance was found to be associated with increasing MIC for CHX. This substantiates the necessity to control the establishment of antimicrobial resistant strains particularly in hospital settings, in conditions such as coronavirus disease 2019 (COVID-19) pandemic which consumption of disinfectants is very high.

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