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Original Article

Antibiofilm and antibacterial activity of diclofenac against clinical enterococcal isolates

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Abstract: Background: Antimicrobial drug resistance is an emerging threat worldwide. Replacing antibiotics with non-antibiotic compounds has been suggested as a way to counteract the magnitude of this problem. We aimed to assess the effect of the non-steroidal anti-inflammatory drug, diclofenac, on the growth of clinical enterococcal isolates, their resistance to the fluoroquinolone antibiotic, ciprofloxacin, and their biofilm forming ability. Methods: We retrieved fifty enterococcal isolates from 36 urine and 14 purulent discharge specimens and determined diclofenac's antimicrobial activity and its effect on their biofilm formation. The effect of exposure of enterococci to diclofenac on the expression levels of the efflux pump genes (*efrA* and *efrB)* and the adherence-related genes (*asa1*, *efaA)* was also studied. Results: We observed that diclofenac inhibited the growth of enterococcal isolates and induced a statistically significant reduction in the expression of *efrA* and *efrB*. However, it failed to reduce either ciprofloxacin resistance rates or its minimal inhibitory concentration. Both ciprofloxacin and diclofenac showed a significant inhibitory effect on enterococcal biofilm formation. Furthermore, diclofenac induced a statistically significant reduction of the expression of *asa1* and *efaA*. Conclusions: The antibacterial activity and the anti-biofilm forming ability of diclofenac demonstrated in this study may provide a promising preventive and therapeutic alternative for medical and dental prosthesis-associated enterococcal infections.

Keywords: Diclofenac, enterococci, ciprofloxacin resistance, biofilm

Introduction

Enterococci constitute the major cause of community and health-care associated infections [1]. The ubiquitous distribution of the genus as normal intestinal flora and the extensive use of broad-spectrum antibiotics and invasive medical devices are major factors contributing to the emergence of resistance of enterococci to the last resort antibiotics, hence the treatment of multi-drug resistance (MDR) enterococcal infections has become a challenging clinical crisis [2, 3].

Ciprofloxacin is a fluoroquinolone antibiotic that targets DNA gyrase and topoisomerase. It has been commonly used for the management of urinary tract infections (UTIs) caused by *Enterococcus faecalis*, resulting in the emergence of fluoroquinolone-resistant enterococci. Quinolone resistance in enterococci is often associated with several mechanisms including mutational alteration of the target and externalization of the antibiotic through over-expression of efflux pumps [3]. EfrAB belongs to one of the multidrug efflux transporters called the ATP-binding cassette (ABC) superfamily which is identified in *E. faecalis* as well as in *E. faecium* [4]. Drug resistance occurs only when *efrA* and *efrB* genes are expressed together [5].

Bacterial biofilm is a population of cells that irreversibly adheres on different biotic and abiotic surfaces [6]. The presence of bacterial biofilm, including enterococcal biofilm, correlates with device-associated infections such as catheter-associated UTIs [7], periodontitis and peri-implantitis causing implant failure [8]. Different studies have described associations between genes coding for adhesion factors and biofilm

development [9]. Amongst these factors, *E. faecalis* endocarditis antigen A (EfaA) and aggregation substance have been studied. The role of *EfaA* gene in the attachment of *E. faecalis* to heart cells has been recognized in cases of endocarditis [10]. It has also been found in *E. faecalis* strains isolated from urine, blood, pleural fluid, peritoneal fluid, eye discharge and endotracheal tubes [11]. Aggregation substance is encoded by a plasmid gene *asa1* and mediates attachment to the host's epithelial cells, inducing biofilm formation [12]. Enterococcal isolates having *asa1* or *efaA* genes have been found to produce significantly more biofilms than negative isolates [13]. Because of the role of biofilms in bacterial virulence and antibiotic resistance, biofilm prevention/elimination has been widely studied. Sub-inhibitory concentrations of antibiotics have been used in order to change the physico-chemical characters and the outer surface structure of enterococcal cells, and to reduce attachment of bacteria to synthetic substrates [14].

To overcome the problem of emergence of antimicrobial drug resistance, substitution of antibiotics with non-antibiotic drugs that demonstrate antibacterial activity is recommended [15]. Diclofenac, a non-steroidal anti-inflammatory drug (NSAID) with a potent analgesic and anti-pyretic effect, has exhibited profound antibacterial effect against both Gram-positive and Gram-negative organisms. The antibacterial effect of this drug is mediated by its phenothiazine compound. The antibacterial effect of phenothiazines is strongly attributed to the halogen groups located in their basic tricyclic ring structure [16-19]. These findings recommend diclofenac as a potent non-antibiotic antibacterial agent [19]. Furthermore, it has been found to decrease biofilm production by some bacterial pathogens including enterococci [20].

There is a paucity of researches that studied the effect of diclofenac on bacterial growth, biofilm formation as well as its effect on antibiotic susceptibility. Therefore, the present study was carried out to evaluate the effect of diclofenac on the growth, ciprofloxacin resistance and biofilm forming ability of enterococcal clinical isolates, as well as its effect on the expression of efflux pump genes (*efrA* and *efrB*) and adherence-related genes (*asa1* and *efaA*) in enterococci.

Methods

Clinical bacterial isolates

This experimental study involved 50 previously identified enterococcal isolates recovered from clinical specimens from Kasr-El-Aini Clinical Laboratories during the period from December 2017 to April 2018. The enterococcal isolates were retrieved from 36 urine samples and 14 purulent discharge samples. An informed consent was obtained from each participant. The study was approved by the Research Ethics Committee of the Institutional Review Board, Faculty of Medicine, Cairo University.

Agar dilution method for diclofenac MICs determination

The minimal inhibitory concentrations (MICs) of diclofenac were also determined by agar dilution method using diclofenac sodium IV infusion ampoules (Novartis, Egypt) (25000 μg/ml) [19, 21].

Briefly, bacterial suspensions of each of the 50 isolates were prepared equivalent to 0.5 McFarland, and 1 μL of each suspension was inoculated on each of ten Mueller Hinton agar plates containing serial dilutions of diclofenac (ranging from 800 to 1.5625 μg/ml). After incubation at 37°C for 24 hours, diclofenac MICs were determined as the lowest concentration of the drug at which there is no visible growth of the organism.

Agar dilution method for ciprofloxacin MICs determination

The MICs of ciprofloxacin were determined by agar dilution method using ciprofloxacin IV infusion vials (2000 μg/ml) (Amriya pharmaceutical industries, Egypt) according to the Clinical and Laboratory Standards Institute guidelines. Briefly, 1 μL of bacterial suspensions equivalent to 0.5 McFarland standard of turbidity of each of the 50 enterococcal isolates was inoculated on Mueller Hinton agar (Oxoid, UK) plates that contain serial dilutions of ciprofloxacin ranging from 64-0.125 μg/ml. Isolates with MIC ≤ 1 μg/ml were considered sensitive to ciprofloxacin, isolates with MIC of 2 μg/ml were considered of intermediate resistance and isolates with MIC of ≥ 4 μg/ml were considered resistant [21].

Table 1. Primer sequences of the studied genes

Gene	Primer sequence	Reference
asa1	Forward primer: GCACGCTATTACGAACTATATGA	*Kafil et al., 2016* [22]
	Reverse primer: TAAGAAAGAACATCACCACGA	
EfaA	Forward primer: TGGGACAGACCCTCACGAATA	*Kafil et al., 2016* [22]
	Reverse primer: CGCCTGTTTCTAAGTTCAAGCC	
EfrA	Forward primer: TTGGCTTTATGACGCCAGT	*Lerma et al., 2014* [26]
	Reverse primer: ATGCGCGTATTACCCGCAA	
EfrB	Forward primer: TAGTGATGATGTTCTTAATCAA Reverse primer: ATTGACTTGTTTAAAGCCTTCA	*Lerma et al., 2014* [26]
23S rRNA	Forward primer: CCTATCGGCCTCGGCTTAG	*Kafil et al., 2016* [22]
	Reverse primer: AGCGAAAGACAGGTGAGAATCC	

The effect of exposure of enterococcal isolates to diclofenac on their ciprofloxacin susceptibility was determined by testing for ciprofloxacin MICs by agar dilution method. Briefly, bacterial colonies were isolated from plates containing the highest concentration of diclofenac that allowed bacterial growth (sub-inhibitory concentration or half MIC of diclofenac). 1 µL of 0.5 McFarland standard of bacterial suspensions of these colonies were inoculated on Mueller Hinton agar plates containing serial dilutions of ciprofloxacin ranging from 64-0.125 µg/ml.

Enterococcal biofilm formation

Enterococcal isolates were tested for biofilm formation by tissue culture plate method in 96-well polystyrene microtiter plates [22]. Furthermore, enterococcal isolates grown in the presence of sub-inhibitory concentrations (½ MIC) of each of diclofenac and ciprofloxacin (the sub-inhibitory concentrations were determined by agar dilution method) were also examined for their biofilm-forming ability. The optical density of the stained adherent biofilm was obtained using micro ELISA autoreader Stat Fax-2100 (Awareness Technology, USA) at wavelength 570 nm. The interpretation of biofilm-formation was done according to the criteria of *Stepanović et al. (2007)* [23].

Quantitative reverse transcription PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was used to assess the effect of exposure of the enterococcal isolates to diclofenac on mRNA expression of *asa1*, *efaA* by 20 biofilm forming enterococcal isolates, as well as the expression of *efrA* and *efrB* by 20 ciprofloxacin resistant enterococcal isolates. Enterococcal isolates grown on Mueller Hinton agar plates containing sub-inhibitory concentrations of diclofenac (that were determined by agar dilution method) were subjected to total mRNA isolation, subsequent cDNA synthesis and quantitative PCR. RNA extraction was performed using QIAGEN RNeasy Mini extraction kit (Qiagen, USA) according to the manufacturer's instructions. The extracted total RNA was used for cDNA conversion using QuantiTect reverse transcription kit (Qiagen, USA). Amplification was performed using SYBR Green I (Qiagen, USA) according to the manufacturer's instructions. In a reaction DNase-free microcentrifuge tube, 10 µl of Syber green master mix was added to 1 µl of each primer, 3 µl of DNA extract and then 10 µl RNAse-free distilled water was added to complete the reaction volume to 25 µl. A negative control tube was set using 3 µl distilled water instead of the DNA extract. The primer sets used in this study (*Bio Basic* Inc., Canada) are shown in **Table 1**. The prepared reaction mixtures were processed in Applied Biosystems *StepOne* Thermal Cycler with software version 3.1 (Applied Biosystems, USA) for amplification and analysis. The cycling conditions were as follows: one hold cycle at 50°C for 2 min, followed by 40 cycles composed of denaturation at 95°C for 15 sec., annealing at 60°C for 60 seconds, and extension at 72°C for 60 seconds. The expression levels of each investigated gene were normalized to the housekeeping 23S rRNA gene and analyzed using the comparative $2^{-\Delta\Delta CT}$ method.[22] The relative quantity (RQ) of studied genes was compared before and after exposure to diclofenac.

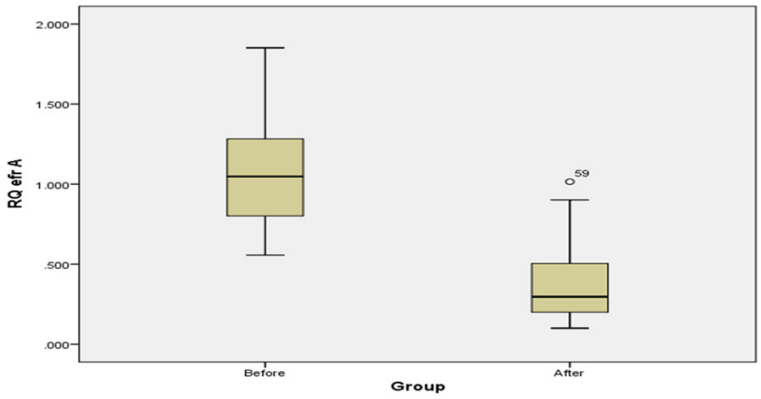

Figure 1. Effect of exposure of enterococcal isolates to diclofenac on *efrA* gene expression. Enterococcal *efrA* gene expression was significantly reduced after exposure to sub-inhibitory concentration of diclofenac (0.38 ± 0.25 *vs.* 1.06 ± 0.36; $P < 0.001$). RQ: relative quantity; Before: before exposure to diclofenac; After: after exposure to diclofenac.

Statistical analysis

Microsoft excel 2013 was used for data entry and the statistical package for social science (SPSS version 24) was used for data analysis. Quantitative data were summarized using simple descriptive statistics (mean and standard deviation) for normal quantitative data, while median and inter-quartile range were used for non-parametric quantitative data. Frequencies were used for qualitative data. Wilcoxon test was used to compare skewed quantitative data, McNemar test was used to compare paired categorical data. Pearson correlation coefficient was used to study correlations between quantitative data. The level of significance was set at probability (P) value ≤ 0.05.

Results

The present study was conducted on 50 previously identified enterococcal clinical isolates retrieved from 36 (72%) urine specimens and 14 (28%) purulent discharge specimens (16% from surgical wounds and 12% from burns).

Antibacterial effect of diclofenac on enteroccal isolates

Out of the 50 enterococcal isolates, the growth of 41 (82%) isolates has been inhibited by dicolfenac at an MIC of 800 µg/ml, while 9 (18%) isolates had a diclofenac MIC of 400 µg/ml.

Effect of diclofenac on ciprofloxacin susceptibility of enterococcal isolates

In this study, out of the 50 enterococcal isolates, 20 (40%) isolates were sensitive to ciprofloxacin, 3 (6%) isolate were intermediately resistant and 27 (54%) isolates were ciprofloxacin resistant. Ciprofloxacin resistance rate among urine sample was 58.3%, while 42.9% of enterococci isolated from purulent discharge specimens were ciprofloxacin resistant.

After exposure of the enterococcal isolates to diclofenac, 21/50 (42%) isolates were sensitive to ciprofloxacin, 2 (4%) isolates were intermediately resistant and 27 (54%) isolates were resistant. Only one of the intermediately resistant isolates became sensitive to ciprofloxacin after diclofenac exposure. There was no statistically significant difference between ciprofloxacin MICs before (median: 16, IQR (inter-quartile range): 1-32) and after exposure to diclofenac (median: 8, IQR: 1-32) ($P = 0.277$).

Effect of diclofenac on the enterococcal efflux pump genes expression

Twenty ciprofloxacin resistant enterococcal isolates were subjected to qRT-PCR in order to measure the expression of *efrA* and *efrB* genes before and after exposure to diclofenac. The expression of *efrA* was significantly lower after diclofenac exposure (0.38 ± 0.25) compared to before diclofenac exposure (1.06 ± 0.36) ($P < 0.001$). Similarly, Exposure to diclofenac induced statistically significant reduction in the *efrB* expression (0.34 ± 0.36) compared to before diclofenac exposure (1.04 ± 0.28) ($P < 0.001$) (**Figures 1**, **2**).

Interestingly, there was no statistically significant correlation between ciprofloxacin MIC values and the expression of each of *efrA* and *efrB* genes either before or after exposure to diclofenac ($r = 0.307$, $P = 0.188$, $r = 0.019$, $P = 0.938$).

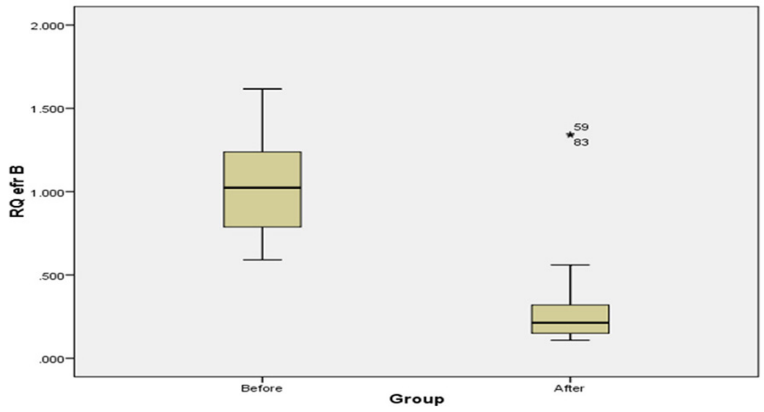

Figure 2. Effect of exposure of enterococcal isolates to diclofenac on *efrB* gene expression. Enterococcal *efrB* gene expression was significantly reduced after exposure to sub-inhibitory concentration of diclofenac (0.34 ± 0.36 *vs.* 1.04 ± 0.28; $P < 0.001$). RQ: relative quantity; Before: before exposure to diclofenac; After: after exposure to diclofenac.

Effect of diclofenac and ciprofloxacin on enterococcal biofilm formation

Out of the 50 enterococcal isolates, 18 (36%) were non biofilm forming, and 32 (64%) were biofilm forming (29 were weak biofilm forming and 3 were moderate biofilm forming). The biofilm forming enterococci were retrieved from 25/36 (69.4%) urine samples and 7/14 (50%) purulent discharge samples.

Exposure of enterococci to ciprofloxacin and diclofenac significantly decreased their biofilm forming ability ($P < 0.001$). After ciprofloxacin exposure, 37/50 (74%) were non biofilm forming and 13/50 (26%) were weak biofilm forming. On the other hand, after diclofenac exposure 46 (92%) were non biofilm forming and 4 (8%) were weak biofilm forming (**Tables 2**, **3**). Interestingly, 19 out of 32 biofilm forming isolates (59.4%) lost their biofilm forming ability after exposure to ciprofloxacin whereas 28/32 (87.5%) isolates lost their biofilm forming ability after exposure to diclofenac.

Effect of diclofenac on the enterococcal adhesion-related genes expression

Twenty biofilm forming enterococcal isolates were subjected to quantitative RT-PCR in order to measure the expression of *asa1* and *efaA* genes before and after exposure to diclofenac. We found that *asa1* gene showed significantly lower *asa1* expression levels after diclofenac exposure (0.39 ± 0.2) compared to before diclofenac exposure (1.05 ± 0.37) ($P < 0.001$). Similarly, exposure to diclofenac induced statistically significant reduction in the *efaA* expression (0.36 ± 0.28) compared to before diclofenac exposure (1.05 ± 0.34) ($P < 0.001$) (**Figures 3**, **4**).

Discussion

We found that diclofenac inhibited the growth of the studied enterococcal isolates at an MIC of 800 µg/ml in 82% of the enterococcal isolates and 8% of the isolates had their diclofenac MIC at 400 µg/ml. Antibacterial activity of diclofenac against enterococci has been shown in previous studies. A lower diclofenac MIC of 50 µg/ml was reported by *Mazumdar et al. (2006)* and *Salem-Milani et al. (2013)* [19]. The MICs reported in the different investigations for diclofenac show great variations, which may be the result of methodological factors, such as the technique and the type of solvent used in the dissolution of the drug. *Johnston et al. (2013)* reported efficient diffusion of a combination of ciprofloxacin and diclofenac loaded on a fibre device used for the treatment of periodontal disease which demonstrated antimicrobial activity against *E. faecalis, Streptococcus mutans* and *Escherichia coli* at pH 4.0 and 6.8 [23].

We investigated the effect of exposure to diclofenac sodium on ciprofloxacin MICs against enterococci but we found no significant difference either in ciprofloxacin resistance rate, where only one of the intermediately resistant isolates became sensitive, or in ciprofloxacin MICs. Although there was no phenotypic change regarding ciprofloxacin resistance rates or MICs, the expression of *efrA* and *efrB* efflux pump genes were markedly reduced after exposure of the enterococcal isolates to diclofenac ($P < 0.001$). A study done by *Lavilla Lerma et al. (2014)* indicated that *efrAB* expression depends to a great extent on the drug used and the type of bacteria. In their study, in spite of the significant changes in mRNA levels of resistance genes, no change in the phenotypic resistance was observed following treatment

Table 2. Effect of exposure of enterococcal isolates to ciprofloxacin on their biofilm forming ability

		After exposure to ciprofloxacin		Total	*P* value*
		Biofilm	Non Biofilm		
Before exposure to Ciprofloxacin	Biofilm	13	19	32 (64%)	< 0.001
	Non Biofilm	0	18	18 (36%)	
Total		13 (26%)	37 (74%)	50 (100%)	

*McNemar test.

Table 3. Effect of exposure of enterococcal isolates to diclofenac on their biofilm forming ability

		After exposure to diclofenac		Total	*P* value*
		Biofilm	Non Biofilm		
Before exposure to Diclofenac	Biofilm	4	28	32 (64%)	< 0.001
	Non Biofilm	0	18	18 (36%)	
Total		4 (8%)	46 (92%)	50 (100%)	

*McNemar test.

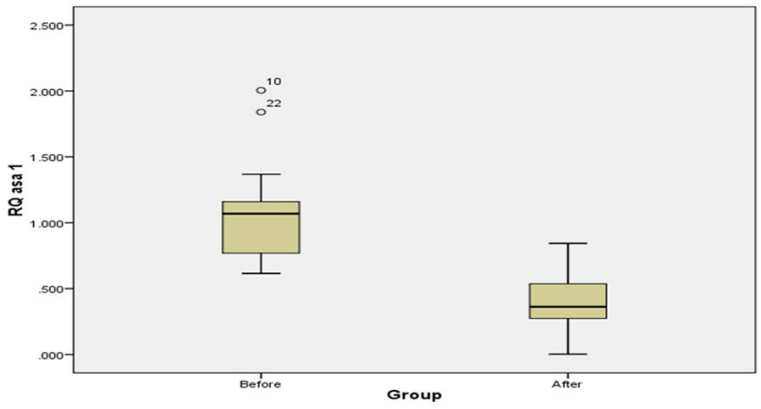

Figure 3. Effect of exposure of enterococcal isolates to diclofenac on *asa1* gene expression. Enterococcal *asa1* gene expression was significantly reduced after exposure to sub-inhibitory concentration of diclofenac (0.39 ± 0.2 *vs.* 1.05 ± 0.37; *P* < 0.001). RQ: relative quantity; Before: before exposure to diclofenac; After: after exposure to diclofenac.

of MDR enterococci with different drugs [25]. This can be attributed to the presence of other mechanisms involved in enterococcal antibiotic resistance, for example, mutation of the target genes *gyrA* and *parC,* or the presence of the *qnr* gene which encodes for a protein that protects DNA gyrase by decreasing DNA binding to the quinolone [3]. In other words, diclofenac can reduce *efrA* and *efrB* gene expression but the isolates remain ciprofloxacin resistant by other mechanisms. This assumption can be confirmed by another finding in the present study which is the absence of correlation between ciprofloxacin MIC values and the expression of each of *efrA* and *efrB* genes.

Several factors may contribute to the reduction in mRNA expression of *EfrAB* in enterococci after diclofenac exposure, for instance, the impairment of the pathway essential for regulation of the expression of efflux pump genes, structural modification of the efflux pump machinery, or lack of the energy necessary for pump activity [26]. The antibacterial effect of diclofenac can be mediated by its chemical structure i.e., chlorine atoms and phenothiazine ring. Diclofenac is composed of a phenyl ring and a secondary amino group; both contain chlorine atoms in their ortho positions. These chlorine atoms may play a pivotal role in conveying antimicrobial activity to this drug [18]. The exact mechanism of antibacterial activity of diclofenac remains to be elucidated. Studies have proposed different mechanism(s) of action like inhibition of bacterial DNA synthesis, impairment of membrane activity [17], anti-plasmid activity [18], and alteration in genes encoding transport/binding proteins, DNA synthesis and cell envelope, down-regulation of efflux pumps [27] and reduced quorum sensing-controlled motility leading to reduced biofilm formation [28]. Furthermore, these non-antibiotic medications may exhibit their antimi-

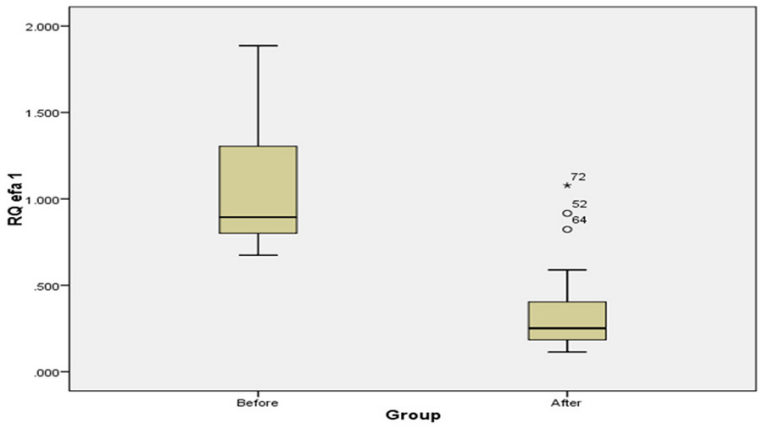

Figure 4. Effect of exposure of enterococcal isolates to diclofenac on *efa1* gene expression. Enterococcal *efa1* gene expression was significantly reduced after exposure to sub-inhibitory concentration of diclofenac (0.36 ± 0.28 *vs.* 1.05 ± 0.34; $P < 0.001$). RQ: relative quantity; Before: before exposure to diclofenac; After: after exposure to diclofenac.

crobial activity via mechanisms distinct from those of known antibiotics, thus promoting antibiotic activity or even reducing antibiotic resistance [29].

Out of the 50 enterococcal isolates involved in this study, 64% were biofilm forming. The biofilm forming enterococci were retrieved from 69.4% of urine samples and 50% of purulent discharge samples. Higher rates of biofilm formation among enterococci were previously reported. Here we showed that exposure of enterococci to sub-inhibitory concentration (half MIC) of each of ciprofloxacin and diclofenac significantly decreased their biofilm forming ability by 59.4% and 87.5% respectively ($P < 0.001$). This finding denotes a decrease in the enterococcal capability to adhere to surfaces. Moreover, we showed that this phenotypic change represented by the significant reduction of the ability of enterococci to form biofilm *in-vitro* after exposure to diclofenac was supported by the significant reduction in the adhesion-related genes (*asa1* and *efaA)* expression levels ($P < 0.001$). Similarly, Diclofenac has significantly enhanced the effect of calcium hydroxide against preformed biofilms of *E. faecalis* on bovine dentin blocks [30]. It also produced significant reduction in the colony forming units count of *E. faecalis,* showing both antibacterial and anti-biofilm activity in an endodontic model [20]. There is a paucity of information about the effect of diclofenac on biofilm production by enterococci. However, sub-inhibitory concentrations of diclofenac have been shown to cause marked inhibition of biofilm formation produced by other microorganisms such as *Staphylococcus aureus* and *E. coli* [31].

To the best of our knowledge, there are no previous studies that investigated the effect of diclofenac on the expression of the adherence-related genes, *asa1* and *efaA,* by enterococci. Though, previous studies have also verified the inhibitory effect of NSAIDs on genes responsible for biofilm formation. The mechanism by which NSAIDs affect biofilm formation is not completely understood. The reduction of components that form the biofilm matrix (such as glycocalyx and teichoic acids), alteration of cell surface hydrophobicity, or inhibition of quorum-sensing components may contribute to the antibiofilm effect of these drugs.

One of the limitations of this study is the small sample size; however, power analysis for repeated measures was performed and revealed that the study power is 100%. Another limitation is the need to investigate the antibiofilm and the antimicrobial effect of diclofenac under various conditions that can be encountered on medical devices such as exposure to other antimicrobials or disinfectants, temperature and pH, as well as *in vivo* parameters including the presence of body fluids, purulent discharge and immunological and inflammatory cells and proteins. Further studies are recommended to elucidate the effect of theses parameters.

An obstacle to the current use of diclofenac as an alternative to the conventional antibiotics, or as an adjuvant drug to the antibiotics, is that, with a few exceptions, the MICs obtained in *in-vitro* tests are above those achieved in serum. However, the fact that diclofenac penetrates efficiently into inflamed tissues and joint fluid producing higher concentrations in these tissues compared to the drug concentrations in plasma should be taken into account. High concentrations of the drug can also be achieved with topical therapy [32]. Alternatively, the use of antimicrobial-coated urinary cath-

eters is a feasible preventive strategy to reduce the risk of catheter-associated UTIs and other device-associated infections [33]. Therefore, coating of medical devices and dental implant-supported prosthesis with diclofenac is a potential approach to combat device-associated infection.

Conclusion

In conclusion, ciprofloxacin resistance was reported in high rate among enterococcal clinical isolates. Accordingly, the use of antibiotics must be restrictive and discriminative to control the emergence of resistant clones. Diclofenac was found to have an *in-vitro* antibacterial activity against enterococci; it inhibited the growth of enterococcal isolates at MIC of 400-800 μg/ml. Although diclofenac induced a statistically significant reduction in the expression of efflux pump genes (*efrA and efr*B), it failed to reduce either ciprofloxacin resistance rate or ciprofloxacin MIC values. These results highlight the fact that quinolones resistance in enterococci can be mediated by various mechanisms other than extrusion of the drug by efflux pump proteins. Additionally, exposure of enterococcal isolates to diclofenac and ciprofloxacin significantly decreased their biofilm formation. Diclofenac also induced a statistically significant reduction in the expression of adhesion-related genes (*asa1* and *efaA*). The findings of this study may provide a new strategy to overcome enterococcal drug resistance and biofilm formation by coating medical devices and dental implant-supported prosthesis with diclofenac. Further *in-vitro* and clinical studies are required to confirm the antibacterial and anti-biofilm activity of diclofenac, taking into consideration the pharmacokinetics and the pharmacodynamics of the drug.

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Disclosure of conflict of interest

None.

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References

[1] Marra A, Dib-Hajj F, Lamb L, Kaczmarek F, Shang W, Beckius G, Milici AJ, Medina I and Gootz TD. Enterococcal virulence determinants may be involved in resistance to clinical therapy. Diagn Microbiol Infect Dis 2007; 58: 59-65.

[2] Raza T, Ullah SR, Mehmood K and Andleeb S. Vancomycin resistant enterococci: a brief review. J Pak Med Assoc 2018; 68: 768-772.

[3] Miller WR, Munita JM and Arias CA. Mechanisms of antibiotic resistance in enterococci. Expert Rev Anti Infect Ther 2014; 12: 1221-1236.

[4] Phillips-Jones MK and Harding SE. Antimicrobial resistance (AMR) nanomachines-mechanisms for fluoroquinolone and glycopeptide recognition, efflux and/or deactivation. Biophys Rev 2018; 10: 347-362.

[5] Shiadeh SMJ, Hashemi A, Fallah F, Lak P, Azimi L and Rashidan M. First detection of efrAB, an ABC multidrug efflux pump in Enterococcus faecalis in Tehran, Iran. Acta Microbiol Immunol Hung 2019; 66: 57-68.

[6] Gupta P, Sarkar S, Das B, Bhattacharjee S and Tribedi P. Biofilm, pathogenesis and prevention-a journey to break the wall: a review. Arch Microbiol 2016; 198: 1-15.

[7] Jamal M, Ahmad W, Andleeb S, Jalil F, Imran M, Nawaz MA, Hussain T, Ali M, Rafiq M and Kamil MA. Bacterial biofilm and associated infections. J Chin Med Assoc 2018; 81: 7-11.

[8] Dhir S. Biofilm and dental implant: the microbial link. J Indian Soc Periodontol 2013; 17: 5-11.

[9] Gao W, Howden BP and Stinear TP. Evolution of virulence in Enterococcus faecium, a hospital-adapted opportunistic pathogen. Curr Opin Microbiol 2018; 41: 76-82.

[10] Reynaud af Geijersstam A, Culak R, Molenaar L, Chattaway M, Røslie E, Peciuliene V, Haapasalo M and Shah HN. Comparative analysis of virulence determinants and mass spectral profiles of Finnish and Lithuanian endodontic Enterococcus faecalis isolates. Oral Microbiol Immunol 2007; 22: 87-94.

[11] Heidari H, Hasanpour S, Ebrahim-Saraie HS and Motamedifar M. High incidence of virulence factors among clinical Enterococcus faecalis isolates in Southwestern Iran. Infect Chemother 2017; 49: 51-56.

[12] Chuang-Smith ON, Wells CL, Henry-Stanley MJ and Dunny GM. Acceleration of Enterococcus faecalis biofilm formation by aggregation sub-

stance expression in an ex vivo model of cardiac valve colonization. PLoS One 2010; 5: e15798.
[13] Kafil HS and Mobarez AM. Assessment of biofilm formation by enterococci isolates from urinary tract infections with different virulence profiles. J King Saud Univ Sci 2015; 27: 312-317.
[14] Hashem YA, Amin HM, Essam TM, Yassin AS and Aziz RK. Biofilm formation in enterococci: genotype-phenotype correlations and inhibition by vancomycin. Sci Rep 2017; 7: 5733.
[15] Ashraf A, Yousri F, Taha N, Abd El-Waly O, Ramadan A and Ismail E. Effect of some non-steroidal anti-inflammatory drugs on growth, adherence and mature Biofilms of Candida spp. Am J Microbiol Res 2015; 3: 1-7.
[16] Martins M, Viveiros M, Kristiansen JE, Molnar J and Amaral L. The curative activity of thioridazine on mice infected with Mycobacterium tuberculosis. In Vivo 2007; 21: 771-775.
[17] Dutta NK, Annadurai S, Mazumdar K, Dastidar SG, Kristiansen JE, Molnar J, Martins M and Amaral L. Potential management of resistant microbial infections with a novel non-antibiotic: the anti-inflammatory drug diclofenac sodium. Int J Antimicrob Agents 2007; 30: 242-249.
[18] Mazumdar K, Dastidar SG, Park JH and Dutta NK. The anti-inflammatory non-antibiotic helper compound diclofenac: an antibacterial drug target. Eur J Clin Microbiol Infect Dis 2009; 28: 881-891.
[19] Salem-Milani A, Balaei-Gajan E, Rahimi S, Moosavi Z, Abdollahi A, Zakeri-Milani P and Bolourian M. Antibacterial effect of diclofenac sodium on Enterococcus faecalis. J Dent (Tehran) 2013; 10: 16-22.
[20] Chockattu SJ, Deepak BS and Goud KM. Comparison of anti-bacterial efficiency of ibuprofen, diclofenac, and calcium hydroxide against Enterococcus faecalis in an endodontic model: an in vitro study. J Conserv Dent 2018; 21: 80-84.
[21] Clinical and Laboratory Standards Institute. Minimal inhibitory concentration breakpoints for Enterococcus spp., 27th edition. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA, USA 2017.
[22] Kafil HS, Mobarez AM, Moghadam MF, Hashemi ZS and Yousefi M. Gentamicin induces efaA expression and biofilm formation in Enterococcus faecalis. Microb Pathog 2016; 92: 30-35.
[23] Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Ćirković I and Ruzicka F. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 2007; 115: 891-899.
[24] Johnston D, Choonara YE, Kumar P, du Toit LC, van Vuuren S and Pillay V. Prolonged delivery of ciprofloxacin and diclofenac sodium from a polymeric fibre device for the treatment of periodontal disease. Biomed Res Int 2013; 2013: 460936.
[25] Lavilla Lerma L, Benomar N, Valenzuela AS, Casado Muñoz Mdel C, Gálvez A and Abriouel H. Role of EfrAB efflux pump in biocide tolerance and antibiotic resistance of Enterococcus faecalis and Enterococcus faecium isolated from traditional fermented foods and the effect of EDTA as EfrAB inhibitor. Food Microbiol 2014; 44: 249-257.
[26] Pagès JM and Amaral L. Mechanisms of drug efflux and strategies to combat them: challenging the efflux pump of Gram-negative bacteria. Biochim Biophys Acta 2009; 1794: 826-833.
[27] Riordan JT, Dupre JM, Cantore-Matyi SA, Kumar-Singh A, Song Y, Zaman S, Horan S, Helal NS, Nagarajan V, Elasri MO, Wilkinson BJ and Gustafson JE. Alterations in the transcriptome and antibiotic susceptibility of Staphylococcus aureus grown in the presence of diclofenac. Ann Clin Microbiol Antimicrob 2011; 10: 30.
[28] Ulusoy S and Bosgelmez-Tinaz G. Nonsteroidal anti-inflammatory drugs reduce the production of quorum sensing regulated virulence factors and swarm in motility in human pathogen Pseudomonas aeruginosa. Drug Res (Stuttg) 2013; 63: 409-413.
[29] Yin Z, Wang Y, Whittell LR, Jergic S, Liu M, Harry E, Dixon NE, Kelso MJ, Beck JL, Oakley AJ and Oakley AJ. DNA replication is the target for the antibacterial effects of nonsteroidal anti-inflammatory drugs. Chem Biol 2014; 21: 481-487.
[30] de Freitas RP, Greatti VR, Alcalde MP, Cavenago BC, Vivan RR, Duarte MA, Weckwerth AC and Weckwerth PH. Effect of the association of nonsteroidal anti-inflammatory and antibiotic drugs on antibiofilm activity and pH of calcium hydroxide pastes. J Endod 2017; 43: 131-134.
[31] Reśliński A, Dąbrowiecki S and Głowacka K. The impact of diclofenac and ibuprofen on biofilm formation on the surface of polypropylene mesh. Hernia 2015; 19: 179-185.
[32] Altman R, Bosch B, Brune K, Patrignani P and Young C. Advances in NSAID development: evolution of diclofenac products using pharmaceutical technology. Drugs 2015; 75: 859-877.
[33] Singha P, Locklin J and Handa H. A review of the recent advances in antimicrobial coatings for urinary catheters. Acta Biomater 2017; 50: 20-40.