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ORIGINAL ARTICLE

Antibacterial and antibiofilm activities of diclofenac against levofloxacin-resistant *Stenotrophomonas maltophilia* isolates; emphasizing repurposing of diclofenac

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ABSTRACT

Background and Objectives: *Stenotrophomonas maltophilia* is an opportunistic pathogen causing nosocomial infections. Diclofenac is an anti-inflammatory drug that is considered a non-antibiotic drug. This study assessed the antibacterial and antibiofilm effects of diclofenac and levofloxacin/diclofenac combination against levofloxacin resistant isolates.

Materials and Methods: Minimum inhibitory concentration was determined using broth microdilution method for levofloxacin, diclofenac, and levofloxacin/diclofenac combination. Biofilm forming capacity and biofilm inhibition assay were determined. Relative gene expression was measured for efflux pump genes; *smeB*, and *smeF* genes and biofilm related genes *rmlA*, *spgM*, and *rpfF* without and with diclofenac and the combination.

Results: Diclofenac demonstrated MIC of 1 mg/ml. The combination-with ½ MIC diclofenac- showed synergism where levofloxacin MIC undergone 16-32 fold decrease. All the isolates that overexpressed *smeB* and *smeF* showed a significant decrease in gene expression in presence of diclofenac or the combination. The mean percentage inhibition of biofilm formation with diclofenac and the combination was 40.59% and 46.49%, respectively. This agreed with biofilm related genes expression investigations.

Conclusion: Diclofenac showed an antibacterial effect against *Stenotrophomonas maltophilia*. The combination showed *in-vitro* synergism, significant reduction in biofilm formation and in the relative level of gene expression. Furthermore, it can potentiate the levofloxacin activity or revert its resistance.

Keywords: Diclofenac; *Stenotrophomonas maltophilia*; Levofloxacin; Biofilm; Synergism

INTRODUCTION

Stenotrophomonas maltophilia is an opportunistic bacterium that causes community and hospital acquired infections. The infections caused by *S. maltophilia* have raised concerns due to increased morbidity and mortality rates (1-4). *S. maltophilia* is furnished with a number of inherent virulence factors. It exhibits a significant level of intrinsic resistance to many antibacterial classes, resulting in limited treatment options; to which it can develop resistance rapidly (5-7). The drug of choice for treating infections of *S. maltophilia* is trimethoprim-sulfamethoxazole. In cases of resistance or allergy, levofloxacin is typically used as the second line antibacterial agent (6, 8). However, resistance to fluoroquinolones has recently become more prevalent due to the acquisition of several resistance mechanisms. The most relevant among these mechanisms is the efflux pump overexpression that can promote fluoroquinolone resistance (espe-

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cially via SmeABC or SmeDEF efflux pumps) (9, 10).

The formation of biofilm by *S. maltophilia* is considered as a key factor in protecting the bacteria against environmental factors, maintaining persistence on medical devices, evading the immune system, and developing antimicrobial resistance. Some of the genes associated with biofilm formation in *S. maltophilia* include, *rmlA, spgM* and *rpfF*. On one hand, *rmlA* encodes glucose-1-phosphate thymidyl transferase and *spgM* encodes a bifunctional enzyme with phosphoglucomutase and phosphomannomutase activities. On the other hand, *rpfF* is an Enoyl-CoA hydratase that serves as a diffusible signal factor (DSF)-based quorum sensing (QS) synthase of the *rpf* (regulation of pathogenicity factors) cluster. It is involved in regulating virulence, and bacterial motility (twitching and swimming), biofilm formation, as well as oxidative stress and antibiotic resistance (5, 11, 12).

Diclofenac is an anti-inflammatory non-steroidal drug that also shows antibacterial effects. This antibacterial effect was referred to as being a phenothiazine compound with halogen substitution in the tricyclic ring structure (13). It has been postulated to have antibiofilm effect against many bacteria (14, 15). Although the exact mechanism of this antibiofilm effect is still not fully understood, it has been suggested that modifying cell surface hydrophobicity or inhibiting components involved in quorum sensing may play a role in the antibiofilm effects (16, 17).

As an approved drug, diclofenac demonstrates the concept of drug repurposing that substitutes the conventional drug discovery process and saves costs and time required for approving new antibiotics. These drugs can provide antibacterial effects on their own or enhance the effects of existing antibiotics as synergistic combinations (18).

The current study aimed to assess the antibacterial and antibiofilm effects of diclofenac against levofloxacin resistant *S. maltophilia* isolates. In addition, it aimed to assess the effects of the combination; levofloxacin/diclofenac both phenotypically and on the level of gene expression. This highlight repurposing of diclofenac which may provide an additional therapeutic option for infections of *S. maltophilia*.

MATERIALS AND METHODS

Bacterial isolates. Isolates of *S. maltophilia* were obtained from different clinical specimens collected from the microbiological laboratories of four hospitals in Alexandria, Egypt during a timeframe of 9 months. Initially, all the isolates were identified using conventional biochemical tests (19), and then confirmed using Vitek-2 (bioMerieux, France). Additionally, *S. maltophilia* ATCC 13637 strain (Oxoid, London, UK) was also included in phenotypic tests and as control in the genotypic investigations.

Antibacterial susceptibility. The susceptibility testing was done using the disc diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI 2022) (20). The antibiotic discs used were minocycline (MIN, 30 µg), trimethoprim-sulfamethoxazole (SXT, 1.25\23.75 µg), and levofloxacin (LEV, 5 µg) discs, which were purchased from Oxoid, Hampshire, UK.

Minimum inhibitory concentration (MIC) determination for diclofenac. MIC of diclofenac (diclofenac sodium IM ampules 75 mg/ml Novartis, Egypt) was determined using the broth microdilution method (20). The MIC was determined against levofloxacin-resistant *S. maltophilia* clinical isolates and the standard strain ATCC 13637. The inoculum of each isolate was adjusted spectrophotometrically to an optical density (OD_{600}) of 0.12-0.13 then diluted to obtain a final concentration of 5×10^5 CFU/ml. Two-fold serial dilutions of diclofenac were prepared using Muller Hinton broth at range of concentrations from 2000 to 3.9 µg/ml. The MIC was visually detected as the well with the lowest diclofenac concentration showing no growth after 20 hours of incubation at 37°C. The test was carried out twice for each isolate. Sub-MIC was denoted as the concentration just below the MIC or ½ MIC.

MIC determination for levofloxacin alone and in the presence of diclofenac sub-MIC. The same methodology was used to detect MIC of levofloxacin for each isolate, where the concentration range of levofloxacin was 64-0.125 µg/ml. Subsequently, diclofenac sub-MIC effect on levofloxacin susceptibility of the isolates was estimated i.e., determining the effect of using the levofloxacin/diclofenac combination. This was also done using broth microdilution method. For each investigated isolate, the MIC of levofloxacin alone was determined and was compared to its MIC when combined with sub-MIC of diclofenac (½ MIC). The fractional inhibitory con-

centration (FIC) was computed using the formula stated by Seukep et al. The results were interpreted to determine if there was synergism, indifference or antagonism (21).

Biofilm assays. The microtiter plate method presented by Hassan et al. (22). was applied to evaluate the biofilm forming capacity of all the studied isolates, with a few minor modifications. Each isolate was incubated in sterile trypticase soy broth (containing 2.5% glucose) in 4 glass tubes; the first tube alone, the second tube with sub-MIC diclofenac, the third tube with sub-MIC levofloxacin and the fourth tube with sub-MIC levofloxacin/diclofenac combination. A negative control well containing broth without bacterial suspension was also included. After incubating at 37°C for 20 hours the inoculum turbidity was adjusted to 1×10^6 CFU/ml. Then, 200 µl of each isolate in each condition was transferred into the microtiter plate, and incubated for 24 hours at 37°C. The contents of all the wells, including free cells and broth were discarded and the wells were washed with phosphate buffer saline (PBS, pH 7.2). The biofilm fixation was done using a 2% sodium acetate solution, and then washed with PBS. The biofilm was stained with a 1% crystal violet solution for 15 minutes, discarded and excess dye was washed away. The dye that stained the biofilm was eluted using 95% ethanol, and the optical density of the eluted stain was measured at 590 nm. This assay was done in triplicates. The average optical density (OD) was computed for each isolate average optical density of the negative control (ODc) was determined. The biofilm forming capacity was interpreted according to Hassan et al. (22). Additionally, the percentage inhibition of biofilm formation with sub-MIC of diclofenac, levofloxacin and the combination was quantified using the formula declared by Lopes et al. (23).

Minimum biofilm inhibitory concentration (MBIC). The same microtiter plate method (22) was used to determine MBIC for diclofenac and levofloxacin with slight modifications. Twofold serial dilutions of diclofenac and levofloxacin were prepared using TSB broth with concentration ranges of 2000 to 3.9 µg/ml and 64 to 0.125 µg/ml, respectively. The inoculum turbidity was adjusted to 1×10^6 CFU/ml as previously described. The microtiter plates were then incubated for 24 hours at 37°C. The biofilm fixation, staining and elution of the stain was done as previously described. A negative control well containing broth without bacterial suspension was also included. The eluted stain was read using an ELISA plate reader at OD590 nm. The MBIC was defined as the lowest concentration that showed a significant reduction in readings compared to the control wells at OD590 nm (24).

Quantitative reverse transcription PCR for gene-expression. The effect of diclofenac and the combination of levofloxacin/diclofenac on the expression of biofilm related genes- *rmlA*, *spgM* and *rpfF*- and 2 efflux pumps of the RND family; SmeABC and SmeDEF (that corresponds *smeB* and *smeF* genes, respectively), were investigated using Real-time PCR. Each isolate was inoculated into fresh LB broth alone, with sub-MIC diclofenac and with sub-MIC of levofloxacin/diclofenac combination. Using PureLink™ RNA Mini Kit from Invitrogen (Thermo Fisher Scientific, California, USA), the total RNA was extracted from the cells in the log phase. The total extracted RNA was measured using Nano-drop spectrophotometer (Thomas Scientific, USA). The High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems™ (Thermo Fisher Scientific, California, USA) was utilized for synthesizing cDNA. Expression levels were estimated using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, California, USA), and the PCR thermal cycler Applied Biosystem StepOne ™ instrument (Thermo Fisher Scientific, California, USA). Table 1 includes a list of the primers used. All the primers included in this study were supplied by Invitrogen (Thermo Fisher Scientific, California, USA). Normalization of the genes expression levels was done using the house-keeping gene *rpoB* gene (25). The expression levels of the biofilm genes of the treated isolates were compared to those of the untreated isolates. While the expression levels of the efflux pumps genes were compared to the standard strain ATCC 13637 (Oxoid, London, UK) (26). The relative expression levels were detected according to $2^{-\Delta\Delta Ct}$ method (25, 26). Each assay was independently done in triplicate. For efflux pump genes the term overexpression was denoted when the relative expression level was >1 (26).

Statistical analyses. IBM SPSS version 20.0 was used for data analysis. (IBM Corporation, Armonk, NY). The significance of the obtained results was determined at the 5% level. The tests included were Chi-square test (for 2 group comparison), Shapiro-Wilk

Table 1. Primers sequences used in this study

Primer	Nucleotide Sequence (5'–3')	Target	Annealing temperature in °C	Product size	Reference
rpfF (F)	GCAGAAGACCAACGTCGGCAAG	*rpfF*	57	1140 bp	(27)
rpfF (R)	CTTCCTAGGCGACGATGGTGTG				
rmlA (F)	CGGAAAAGCAGAACATCG	*rmlA*	49	1222 bp	(27)
rmlA (R)	GCAACTTGGTTTCAATCACTT				
spgM (F)	ATACCGGGGTGCGTTGAC	*spgM*	53	2750 bp	(27)
spgM (R)	CATCTGCATGTGGATCTCGT				
smeB (F)	ACCGCCCAGCTTTCATACAG	*smeABC*	53	944 bp	(28)
smeB (R)	GACATGGCCTACCAGGAACAG				
smef (F)	TCGTCCAGGCTGACATTCAA	*smeDEF*	53	1061 bp	(28)
smef (R)	AACGCGGATCGTGATATCG				
rpoB (F)	AGGAAATGCTGACGGTGAAG	*rpoB*	50	3637 bp	(25)
rpoB (R)	ACGAGCACGTTGAAGGATTC				

test (checking the normality of continuous data), and Kruskal Wallis test (assessing for quantitative variables with non-parametric distributions). Also, the Post Hoc (Dunn's multiple comparisons test) was utilized for pairwise comparisons.

RESULTS

Bacterial isolates and antibacterial susceptibility. Sixty clinical isolates of *S. maltophilia* were collected from microbiological laboratories in different hospitals in Alexandria, Egypt. The samples included blood samples, sputum samples, bronchoalveolar lavage (BAL) samples and wound swabs. The results of the susceptibility test showed that 24/60 isolates (40%) were resistant to levofloxacin. The levofloxacin sensitive and intermediate-resistant isolates were excluded from subsequent investigations as the levofloxacin resistant isolates were the main concern. For minocycline, 3/24 (12.5%) were resistant, 18/24 (75%) were intermediate and 3/24 (12.5%) were sensitive. Whereas for trimethoprim-sulfamethoxazole 13/24 isolates (54.2%), 11/24 (45.8) were intermediate and none of the isolates were sensitive.

MIC determination for diclofenac against levofloxacin-resistant isolates. The MIC of diclofenac against all the levofloxacin-resistant isolates and *S. maltophilia* ATCC 13637 strains was 1 mg/ml. The sub-MIC was considered as the concentration just below the MIC or ½ MIC (500 µg/ml), which was used in the subsequent investigations.

MIC determination for levofloxacin alone and with diclofenac sub-MIC. The range of levofloxacin MIC against the 24 *S. maltophilia* isolates was from 8 to 16 µg/ml which based on CLSI guidelines confirms resistance of these 24 isolates to levofloxacin. The MIC of levofloxacin against *S. maltophilia* ATCC 13637 strains was 0.5 µg/ml. When combined with sub-MIC (½ MIC) diclofenac, the levofloxacin MIC dropped and ranged from 0.25-05 µg/ml producing a MIC fold decrease corresponding to 16-32-fold. The levofloxacin/diclofenac combination had FIC ranged from 0.016 to 0.06 in all the isolates and *S. maltophilia* ATCC 13637 strain; which corresponded synergism.

Biofilm assays. The investigated isolates for biofilm forming capacity showed that 12 isolates (50%) were strong producers and 12 isolates (50%) were moderate producers. There were no weak or non-forming biofilm among the tested isolates. After subjecting the isolates to sub-MIC of diclofenac, levofloxacin and the combination of both, the biofilm forming capacity was significantly decreased ($p < 0.001$) (Table 2). Meanwhile, the mean values of biofilm formation percentage inhibition with sub-MIC of diclofenac, levofloxacin and the combination were 40.59% ± 17.39, 30.23% ± 17.58 and 46.49% ± 18.70 (mean ± SD), respectively.

Determination of MBIC of diclofenac and levofloxacin. The MBIC range for levofloxacin against the clinical isolates was determined to be 64-32 µg/ml, and 1 mg/ml for *S. maltophilia* ATCC 13637. Conversely, the MBIC range for diclofenac against

Table 2. Comparison between the isolates according to biofilm forming capacity

	Control (n = 24)	levofloxacin (n = 24)	Diclofenac (n = 24)	combination (n = 24)	χ^2	p
Weak biofilm producers	0 (0%)	4 (16.7%)	8 (33.3%)	12 (50%)		
Moderate biofilm producers	12 (50%)	12 (50%)	16 (66.7%)	12 (50%)	35.856*	<0.001*
Strong biofilm producers	12 (50%)	8 (33.3%)	0 (0%)	0 (0%)		
p0		^{MC}p=0.106	^{MC}p<0.001*	<0.001*		
Sig. bet. Groups		$^{MC}p_1$=0.00 *, $^{MC}p_2$=0.002*, p_3: 0.242				

χ^2: Chi square test MC: Monte Carlo

p: p value for comparing between the different studied groups

p_0: p value for comparing between Control and each other groups

p_1: p value for comparing between levofloxacin and diclofenac

p_2: p value for comparing between levofloxacin and levofloxacin in combination

p_3: p value for comparing between diclofenac and levofloxacin in combination

*: Statistically significant at $p \leq 0.05$

the clinical isolates was found to be between 62.5-250 µg/ml, with an MBIC of 125 µg/ml for *S. maltophilia* ATCC 13637. To compare the effects of diclofenac on growth inhibition and biofilm inhibition, the ratio of MIC to MBIC was calculated. Diclofenac exhibited a range of 4-16-fold decrease in the required concentration for biofilm inhibition (MBIC) compared to that required for growth inhibition (MIC).

Investigation of biofilm related genes expression. The expression of the biofilm related genes (*rpfF, rmlA* and *spgM*) in 24 *S. maltophilia* isolates was measured with and without the sub-MIC of diclofenac and levofloxacin/diclofenac combination. It was observed that the sub-MIC of both diclofenac and levofloxacin/diclofenac combination significantly decreased the expression level ($p < 0.001$) of the genes *rpfF, rmlA* and *spgM* (Fig. 1).

Investigation of efflux pump over expression. Out of the 24 levofloxacin resistant isolates, 16 (66.67%) isolates showed overexpression in *smeB* whereas only 4 (16.7%) isolates showed overexpression in *smeF*. Concerning *smeB* gene, all the 16 isolates had a significant decrease in the expression level when exposed to sub-MIC diclofenac as well as in presence of levofloxacin/diclofenac combination. ($p<0.001$) (Fig. 2).

Similarly, for the *smeF* gene, the 4 isolates that showed overexpression had a significant decrease in expression level with sub-MIC diclofenac (Fig. 2). Interestingly, the isolates that did not show overexpression in both genes also undergone a decrease in the level of expression when exposed to either diclofenac or the combination (Fig. 2).

DISCUSSION

S. maltophilia is a pathogen known for its potential to cause healthcare-related infections, with significant level of intrinsic resistance to many antibacterial classes, resulting in limited treatment options; to which it can develop resistance rapidly (6-8). One strategy proposed to control the uncontrollable emergence of antimicrobial resistance is to replace antibiotics with non-antibiotic drugs. Diclofenac, an anti-inflammatory drug, also has antibacterial and antibiofilm effects and so considered a non-antibiotic drug. Moreover, it is regarded as a prime example of drug repurposing, offering a rapid, safe and cost-effective therapeutic alternative (18).

The current study aimed to assess antibacterial and antibiofilm effects of diclofenac and levofloxacin/diclofenac combination. Specifically, the study focused on levofloxacin resistant *S. maltophilia* isolates, both phenotypically and at the level of gene expression; which produces an emphasis for diclofenac drug repurposing providing an additional therapeutic option for *S. maltophilia* infections.

This study included 60 clinical isolates from different sample types; blood, respiratory and wound samples. The isolates were collected from laboratories of different hospitals in Alexandria, Egypt. Out of the 60 isolates 24 (40%) were resistant to levofloxacin

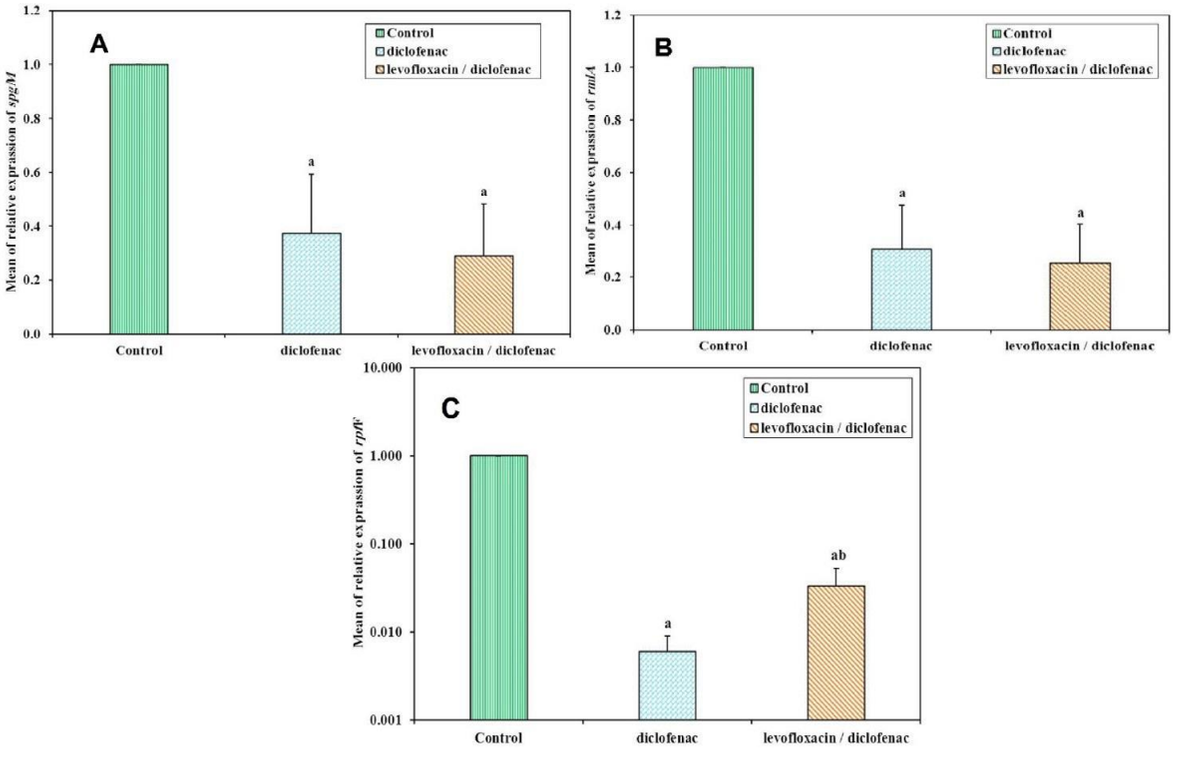

Fig. 1. Comparison between the effect of exposure of the isolates to diclofenac and levofloxacin/diclofenac combination on the gene expression of biofilm related genes.

½ MIC diclofenac and levofloxacin/diclofenac combination produced a significant reduction in the expression levels of biofilm investigated genes. The data shown represent the means ± standard deviations. Pairwise comparisons between 2 groups were done using Post Hoc Test (Dunn's for multiple comparisons test-$p < 0.001$), with statistical significance at $p \leq 0.05$.

A represents the results of *spgM* gene, B represents the results of *rmlA* gene and C represents the results of *rpfF* gene.

a: Significant compared with the control isolates (untreated).

b: Significant compared with the diclofenac.

which were our concern and undergone all the subsequent investigations.

Diclofenac demonstrsted an antibacterial effect against levofloxacin resistant isolates and the *S. maltophilia* ATCC 13637 strain where the MIC was 1 mg/ml. Laundy et al. also observed an antibacterial effect of diclofenac against *S. maltophilia* isolates and the ATCC 13637 strain with MIC values ranging from 0.8-1.6 mg/ml (29).

The effect of diclofenac sub-MIC on levofloxacin MIC was assessed by applying the levofloxacin/diclofenac combination to *S. maltophilia* clinical isolates and the ATCC 13637 strain. Surprisingly, all of the levofloxacin resistant isolates showed a 16 to 32-fold decrease in MIC where all of them reverted their sensitivity to levofloxacin. With diclofenac sub-MIC, the levofloxacin MIC ranged from 0.25-0.5 µg/ml. Additionally, the *S. maltophilia* ATCC 13637 strain also showed a 2-fold decrease in levofloxacin MIC with diclofenac. This combination showed a promising synergism with a FIC range of 0.016 to 0.06.

The effect of diclofenac in combination with levofloxacin and/or other fluroquinolones on different Gram-positive or Gram-negative bacteria was previously studied. Mohamed et al. found synergism between diclofenac and levofloxacin or ciprofloxacin against clinical isolates of *Acinetobacter baumannii* (30). Whereas for Gram positive bacteria; Riordan et al. demonstrated that diclofenac increased susceptibility of *Staphylococcus aureus* to ciprofloxacin, ofloxacin and norfloxacin, thereby improving the antibiotics susceptibility. This was achieved by altering the expression of regulatory and structural genes associated with cell wall biosynthesis and down-regulating some efflux pump genes (31).

Nevertheless, Li X et al. indicated in a study on the *Esherichia coli* ATCC 700926 strain that diclofenac promoted antibiotic resistance at a concentration of

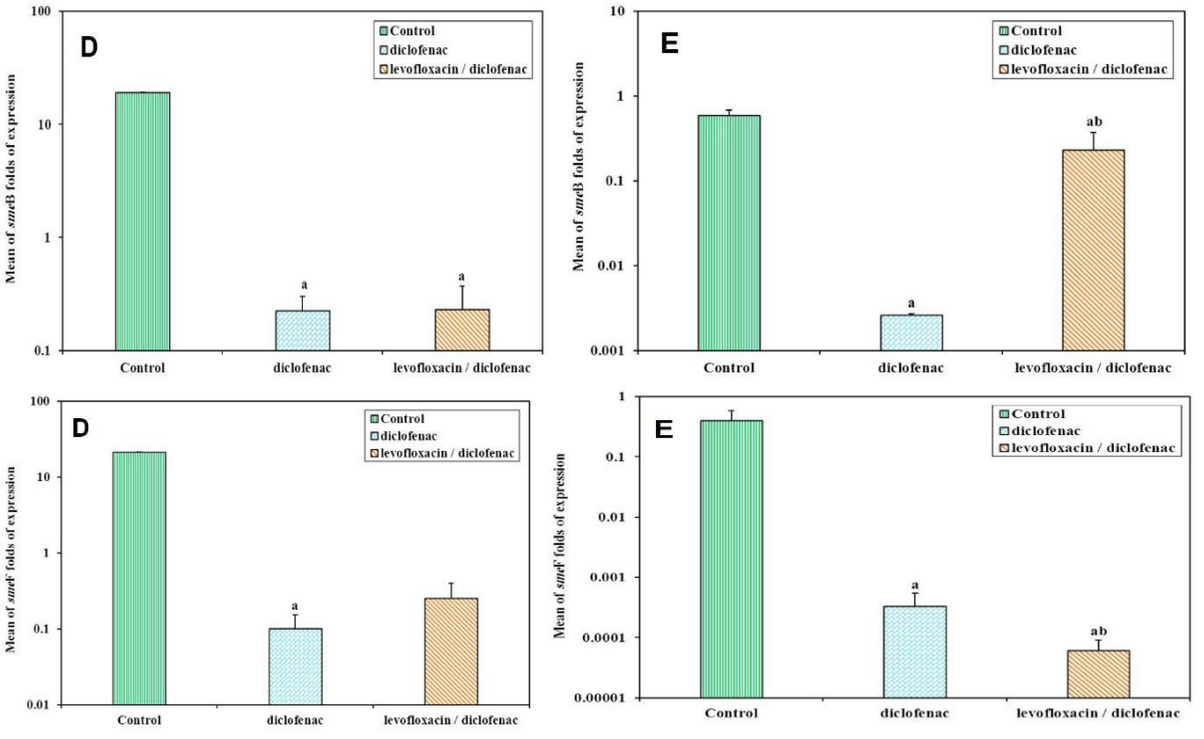

Fig. 2. Comparison between the effect of exposure of the isolates to diclofenac and levofloxacin/diclofenac combination on according to *smeB* or *smeF* fold of expression

The data shown represent the means ± standard deviations. Pairwise comparisons between 2 groups were done using Post Hoc Test (Dunn's for multiple comparisons test- $p < 0.001$), with statistical significance at $p \leq 0.05$.

D represents the effects in isolates that showed overexpression

E represents the effects in isolates that did not show overexpression

a: Significant compared with the control isolates (untreated).

b: Significant compared with the diclofenac.

10 µg/ml as it can enhance the mutation rate through inhibiting the antioxidant system, SOS system. Conversely, higher diclofenac concentration (100 µg/ml) decreased the mutation frequency but increased the resistance of mutants (32). Further studies are required to assess similar effects of diclofenac on *S. maltophilia* clinical isolates.

One of the mechanisms of *S. maltophilia* resistance to fluroquinolones is overexpression of SmeABC and/or SmeDEF efflux pumps (28, 33, 34). Therefore, this study investigated the gene expression of *smeB* and *smeF* that corresponded to SmeABC and SmeDEF efflux pumps, respectively. Out of the 24 levofloxacin-resistant isolates, 66.67% showed overexpression of *smeB* while 16.7% of the isolates showed overexpression of *smeF*. Cho et al. also reported *smeB* overexpression in 63.6% and *smeF* overexpression in 57.5% of their investigated *S. maltophilia* clinical isolates (35). Additionally, Chang et al. found that 59% of the *S. maltophilia* isolates overexpressed *smeB* while 31% overexpressed *smeF* (28).

Diclofenac sub-MIC showed a decrease in the expression level of the genes *smeB* and *smeF* for isolates with both overexpressed and non-overexpressed pumps. For the isolates with overexpressed pumps diclofenac sub-MIC significantly decreased the relative expression to the extent that they were no longer considered overexpressed (relative expression became <1). Similar findings were also observed with the levofloxacin/diclofenac combination, which is consistent with the phenotypic synergistic results of the combination.

According to Laudy et al. phenotypic tests (using an efflux pump inhibitor), diclofenac was suggested to be a substrate of *S. maltophilia* efflux pumps. Still, it did not induce quinolone resistance (29). However, the antibacterial effect of diclofenac was mainly attributed to its ability to inhibit DNA synthesis (36),

which may explain its synergistic effect with levofloxacin. Other studies also related the antibacterial effect of diclofenac to alteration of membrane activity (36), impairment of genes that express transport proteins or even down regulation of efflux pumps (31). Our results support that diclofenac reduces the expression of efflux pump genes.

Diclofenac has been found to have an antibiofilm effect against several bacteria including *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* (16, 17, 37). In this study, 50% of the 24 isolates investigated were strong producers of biofilm while 50% were moderate producers. After exposing the isolates to sub-MIC of diclofenac, levofloxacin and their combination, the mean percentage inhibition of biofilm formation was 40.59%, 30.23% and 46.49%, respectively. Additionally, a significant decrease of the biofilm forming capacity was demonstrated ($p < 0.001$).

The resistance of biofilm cells to levofloxacin was higher than that of planktonic cells. Levofloxacin exhibited a range of MIC values from 8-16 µg/ml and a range of MBIC values from 64-32 µg/ml, resulting in a MBIC/MIC ratio ranging from 2-8. This ratio indicates that the concentration required to inhibit biofilm formation is 2 to 8 times higher than the concentration required to inhibit growth, confirming the increased resistance of biofilm cells to levofloxacin. The same trend was observed in the *S. maltophilia* ATCC 13637, which showed a 2-fold increase in MBIC compared to MIC. Meanwhile, diclofenac showed a 4-16-fold decrease in its MBIC compared to its MIC. Since, diclofenac MIC values were higher than the concentration required to inhibit biofilm formation (MBIC), therefore the reduction in biofilm formation is not due to the bactericidal effect of diclofenac. Consequently, the MBIC actually reflects diclofenac ability to inhibit biofilm formation (38).

The phenotypic results agreed with the expression level of biofilm-related genes; where the expression of *rpfF, rmlA* and *spgM* genes was significantly decreased ($P < 0.001$) with sub-MIC of both diclofenac and levofloxacin/diclofenac combination. Therefore, the biofilm phenotypic and genotypic results indicate that the presence of diclofenac or levofloxacin/diclofenac combination may contribute to a decrease in *S. maltophilia* adherence to biotic and abiotic surfaces. Also, the antibiofilm effect of diclofenac may be attributed to reducing motility or inhibiting quorum sensing (16).

A limitation of this study is that the effective in-vitro concentration of diclofenac (0.5mg/ml) detected is higher than the maximum plasma concentration (15). Although a similar diclofenac concentration has been reported before (39), yet this concentration (0.5mg/ml) may result in adverse events or even toxicity. Therefore, careful management is required for therapeutic use. Hence, studies are urgently required to assess the safety of repurposing diclofenac in *in-vivo* models.

Alternatively, the sub-MIC (0.5mg/ml) of diclofenac alone or in combination with levofloxacin can be used topically in attempt to reduce biofilm formation in wounds that may not be associated with systemic toxicity (39). Furthermore, it can be used in coating medical devices such as catheters, endoscopes and ventilators to reduce infections associated with the colonization of medical devices and surfaces with biofilms. However, *in vivo* studies are still required to determine the safety of topical application of diclofenac.

Another important concern is the drug-drug interaction between the combination components; diclofenac and levofloxacin. A previous study has demonstrated that diclofenac causes a mechanism-based inactivation of cytochrome p450 3A4 (CYP3A4) (40), although, it has not been identified as one of the potent inhibitors. Additionally, quinolones are primarily metabolized by CYP2C9, not by CYP3A4 (41). Thus, in this combination, diclofenac is not expected to elevate the plasma concentrations of levofloxacin to toxic levels that could cause seizures.

Finally, this *in-vitro* study highlights the effect of diclofenac as non-steroidal anti-inflammatory drug that has proven to have antibacterial and antibiofilm effects. Still, many other studies demonstrated other non-steroidal anti-inflammatory drugs-such as ibuprofen (42), piroxicam, acetylsalicylic acid (39), celecoxib (43) and etodolac (44) to have antibacterial and antibiofilm effects in concentrations within the range of those in human pharmacokinetics studies. This suggests that these drugs could be repurposed as an adjuvant therapy for biofilm related infections.

CONCLUSION

Antimicrobial treatment options for *S. maltophilia* infection are scarce which include fluoroquinolones such as levofloxacin. However, resistance to fluoro-

quinolones has been increasing. In this regard, diclofenac has showed a promising antibacterial and antibiofilm effects against *S. maltophilia*. The diclofenac/levofloxacin combination showed in-vitro synergism, significant reduction in biofilm formation and in the gene expression levels. The findings of this *in-vitro* study shed the light on the use of diclofenac as an effective alternative treatment for *S. maltophilia* infections. Furthermore, it can potentiate the activity of levofloxacin or revert its resistance.

However, further studies on diclofenac are still required to understand the underlying mechanism behind its antibiofilm activity. It is important to conduct clinical and *in-vivo* studies, taking into consideration the toxicological properties of diclofenac. Additionally, these studies should asses the possible use of lower concentrations of diclofenac that can still maintain its antibiofilm activity *in-vivo*. Other research studies could also explore the use of nanoparticles for targeted delivery of levofloxacin/diclofenac combination, aiming to reduce toxicity.

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