International Journal of Medical Science and Health Research

Vol. 2, No. 05; 2018

ISSN: 2581-3366

*sep*A gene is positively regulated in fluoroquinolones exposed MRSA-biofilm

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Abstract

Efflux pumps appear to be essential in bacterial bio films and have been reported as one of the mechanisms responsible for the antimicrobial resistance in bio film structures. In this study, we tested the response of MRSA clinical isolates to sub inhibitory concentrations of ciprofloxacin associated with reserving (an efflux pump inhibitor). Bio film formation decreased in presence of ciprofloxacin and reserving. On the other hands expression of sep Agene (coding for an efflux pump) increased with ciprofloxacin and returns to control levels when bio film was exposed to ciprofloxacin and reserving eat the same time. These results confirms its involvement with bio film formation and suggest sep A as important target for further studies to treat mature bio film.

Keywords: S.aurous; bio film; sep A; efflux pump

Introduction

Multidrug efflux systems have previously been suggested to be important in the intrinsic antibiotic resistance of bio films and have shown to be involved in the ability of various bacteria species to form bio film (Baugh *et al.*, 2014). Due to the fact that multidrug resistant pumps play an important role in the resistance of plank tonic bacteria to antimicrobial agents it seems logical that attachment to surfaces and adopting the bio film mode of growth might be signalling cells to increase the expression of efflux pumps (De Kievit & Iglewski, 2000). Several proteins in the efflux pump systems were, named Nor A, Nor B, Nor C, Mde A, Sep A(Costa *et al.*, 2013; Narui *et al.*, 2002; Truong-bolduc *et al.*, 2006; Yamada *et al.*, 2006) and their role in bacterial bio film requires further investigation. In particular, Sep A is well known as a multidrug efflux pump (Narui *et al.*, 2002) and the study of its role in bacterial bio film is very recent as well its functions in the bio film life cycle are not well characterized (Paharik *et al.*, 2016).

An example of antimicrobial in which efflux pumps' over expression may also play an important role in resistant is fluoroquinolones (Piddock, 2006). Fluoroquinolones have been increasingly

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used as a treatment for infections caused by methicillin resistant *S. aurous* (MRSA) in several countries(Coia *et al.*, 2006).

Efflux pump inhibitors (EPIs) have previously been shown to decrease bio film formation in *S. aurous* (Baugh *et al.*, 2014; Costa *et al.*, 2013; Kourtesi *et al.*, 2013; Trotonda *et al.*, 2008). Due to efflux pump inhibition activity of EPIs as well their ability to block some types of antibiotic resistance in bacteria, a great attention has been given to it in the scientific community (Baugh *et al.*, 2014; Kourtesi *et al.*, 2013). As the presence of the *sep* A gene was already related to bio film in *S. epidermidis* (Paharik *et al.*, 2016), these facts motivated us to investigate the expression of *sep*A in clinical isolates of MRSA in bio film eradication as a multidrug efflux pump using a fluroquinolone with EPI. The aims of this study were to investigate in MRSA clinical isolates: (1) bio film formation with fluoroquinolones sub-inhibitory concentration with and without EPI; (2) minimal bactericidal concentration of fluoroquinolone necessary to eradicate bio film with and without EPI; and (3) expression of *sep* A gene in bio film treated with fluoroquinolone and the effect of EPI under the same condition.

Methods

Four clinical isolates of MRSA ($3{,}0x10^8$CFU per well) were incubated to form bio film overnight at 37 °C in 96-well micro plates with ciprofloxacin in sub-inhibitory concentration. To obtain the sub-inhibitory concentration, we first performed broth micro dilution method according CLSI 2016 and the sub-inhibitory concentration used was half the minimum inhibitory concentration for each isolate. The effects of reserving (EPI) (20 µg/ml) were evaluated by crystal violet staining after 24h. The minimal bactericidal concentration of fluoroquinolone with or without EPI against plank tonic cells was measured according to a previous study (Zhang & Mah, 2008). The minimal bactericidal concentration of fluoroquinolone with or without EPI against bio film was performed according to Frank, et. al., 2007. For RNA extraction, TRI zol method was used, following the manufacturer's protocol and quantified by namedrop. After extraction, 200 ηg were used for treatment with DNA se and c DNA synthesis. Quantitative RT–PCR (q RT–PCR) was performed in triplicate using a SYBR Green q RT–PCR Kit (Applied Byosistem®) for the efflux pumps genes and data were analyzed by $2^{-\Delta ct}$ method.

Results

The presence of EPI with ciprofloxacin decreases significantly ($p<0.01$) the biofilm formation in all clinical isolates tested (Figure 1).

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ISSN: 2581-3366

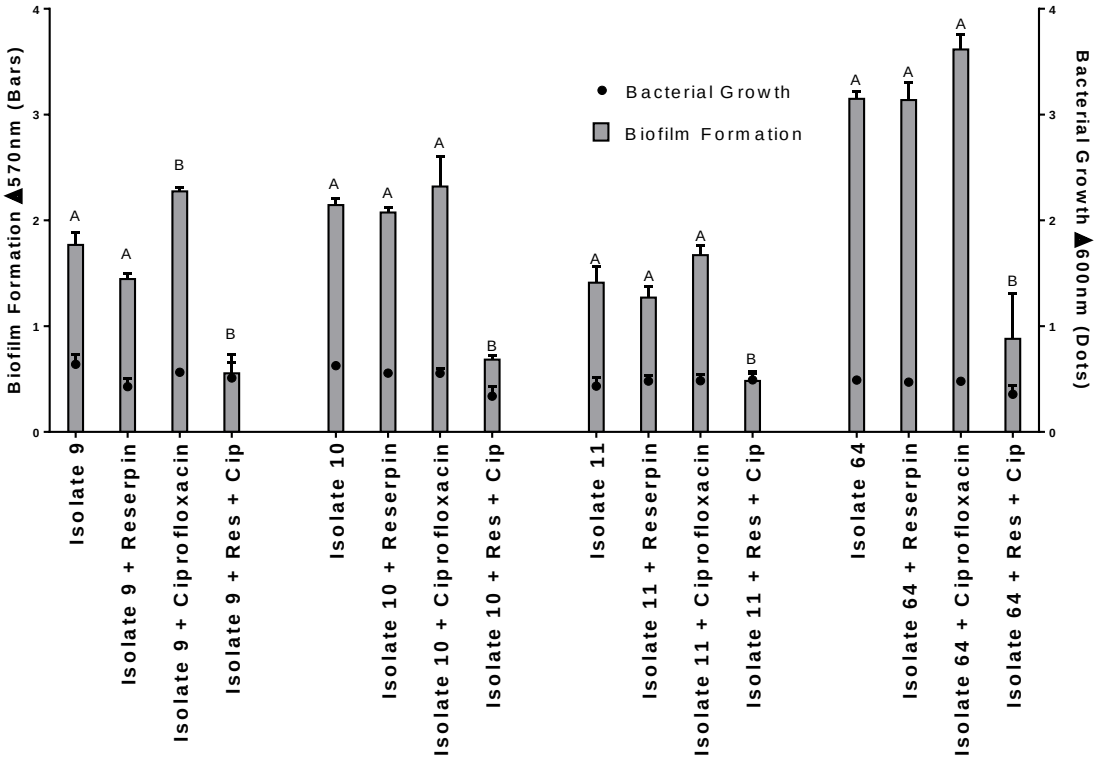

Figure 1. MRSA clinical isolates exposed to sub-inhibitory concentration of ciprofloxacin (CIP) and efflux pump inhibitor (reserving (RES) 20 µg/ml). Data is shown as the mean ±SD from three experimental replicates of three biological replicates. Means with the same letter are not significantly different to control (bacteria without CIP and without RES), as analyzed by one-way ANOVA followed by Turkey muticomparisontest..

As the crystal violet stain in measures the global biomass present in the bio film, MTT assay (colorimetric assay for assessing cell metabolic activity) was performed in order to investigate if the reserving could kill bacteria and it is possible to verify that bacterial death is not the reason for the decrease of bio film formation (data not shown). In Table 1 it was possible to verify a decrease in the concentration of antimicrobial necessary (MBC: minimal bactericidal concentration) to kill the MRSA as well as to eradicate its bio film formation (MBEC: minimal bio film eradication concentration) as a result of EPI addition.

Table 1. In vitro susceptibility testing and bio film susceptibility assay of ciprofloxacin *plus* efflux pump inhibitor against MRSA clinical isolates

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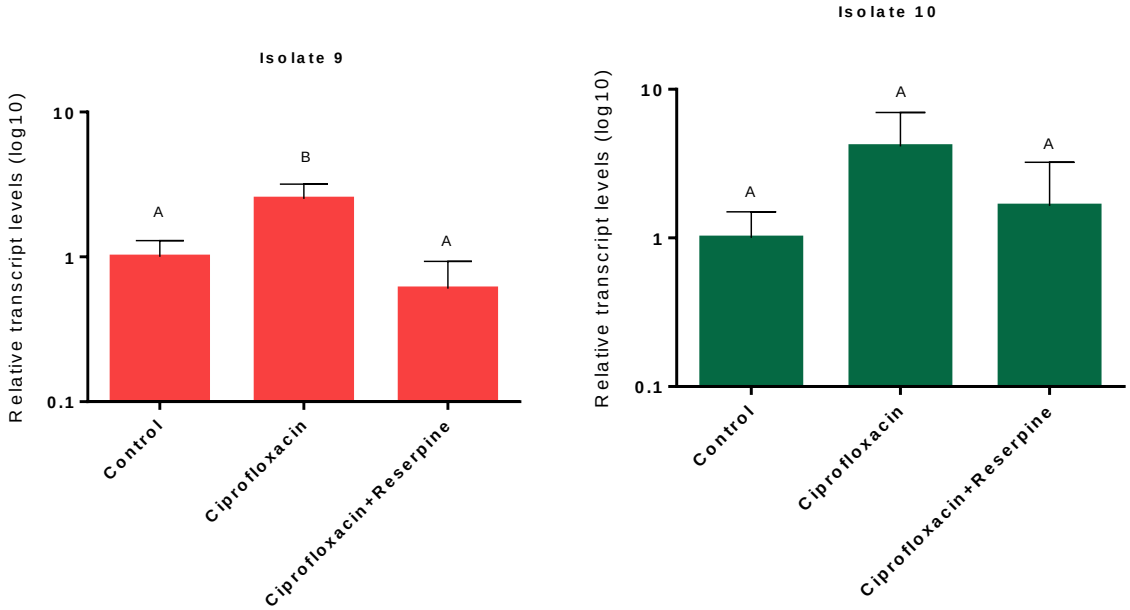
	Ciprofloxacin			
	Without EPI		With EPI	
MRSA strains	MBC (µg/mL)	MBEC (µg/mL)	MBC (µg/mL)	MBEC (µg/mL)
9	128	>512	64	32
10	128	>512	32	4
11	128	>512	32	8
64	128	>512	128	32

Sub-inhibitory concentrations of ciprofloxacin added to mature bio film increased *sep* A relative transcripts levels on 4 clinical isolates of MRSA. When mature bio film was exposed to sub-inhibitory concentrations of ciprofloxacin as well as to an EPI, the expression of the gene returned to control levels (Figure 2).



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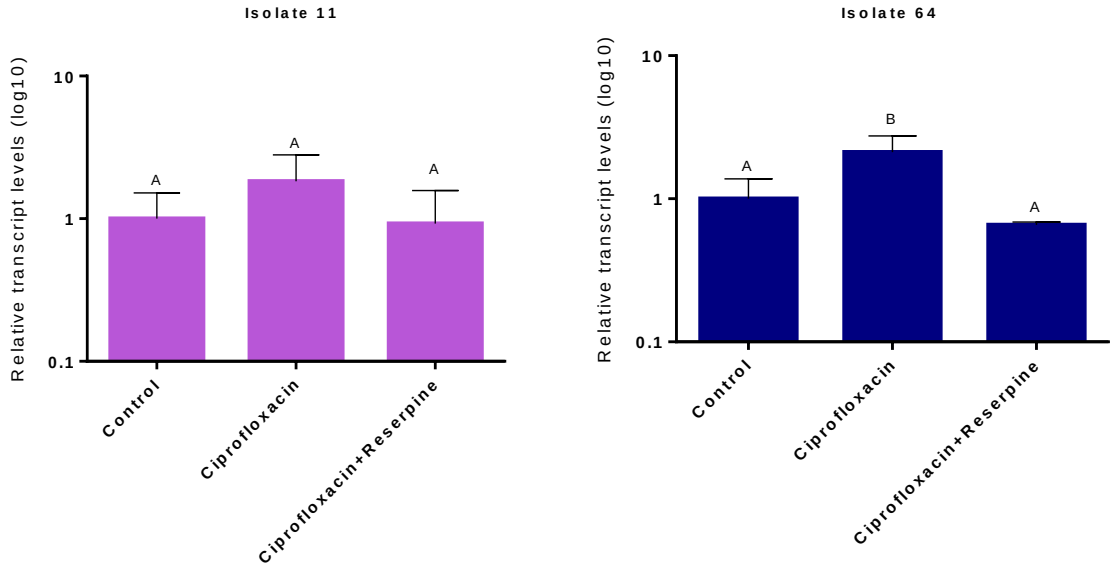

Figure 2. Quantitative real time RT-PCR of sep A gene transcripts in bio film cells of MRSA clinical isolates exposed to sub-inhibitory concentrations of Ciprofloxacin and Ciprofloxacin + Proserpine. The measured quantity of the mRNA in each sample was normalized using *Ct* values obtained from the 16S gene ($2^{-\Delta ct}$). Data is shown as the mean ±SD from three experimental replicates of three biological replicates. Means with the same letter are not significantly different, as analyzed by one-way ANOVA followed by Turkey multicomparison test.

Discussion

A study demonstrated that the bioactive extracts of berry pomace with methicill in significantly reduced MRSA bio film formation on plastic surface by down-regulating the expression of efflux pump including *sep A* genes(Salaheen *et al.*, 2017). In our study we could show that *sep A* is involved at least partially with MRSA-bio film, since the decrease in its expression (when reserving is added to ciprofloxacin - Figure 2) is correlated with the decrease of bio film biomass (Figure 1). This is the first report of this association in *S. aurous* clinical isolates.

Our study shows that bio film formation increases when the isolates are exposed to sub-inhibitory concentrations of ciprofloxacin and decreases when exposed to sub-inhibitory concentrations of ciprofloxacin as well as to an efflux pump inhibitor. These results associated with the decrease of gene expression with ciprofloxacin and EPI suggest *sep*A is one of the multidrug efflux genes which is probably related with bio film formation in these isolates and supported by the previous confirmation of several studies about the necessity of the efflux pumps to maintain the bacterial bio film (Baugh *et al.*, 2014; Gilbert *et al.*, 2002; Kourtesi *et al.*, 2013;

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Kvist *et al.*, 2008; Soto, 2013; Trotonda *et al.*, 2008; Zhang & Mah, 2008). Such data suggest that inhibition of the efflux pump could be a way to make the antimicrobial more effective on the mature bio film. Together these findings shed light on an additional gene, sepA, which plays an important role in *S. aurous* bio film development mechanisms that warrant further investigation.

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