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Glyceryl Trinitrate, a Vasodilating Drug Acts as an Antibiofilm Agent in *Serratia marcescens*

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Abstract

Objectives: *Serratia marcescens* is a gram-negative pathogen of many species. The ability of *S. marcescens* to form biofilms and its potent innate resistance to antimicrobials and cleaning solutions are both essential for its pathogenicity and survival. The present study was conducted to investigate the effect of glyceryl trinitrate (GTN) on the biofilm of *S. marcescens*, as an alternative for antibiotic therapy.

Methods: Different specimens, including ear swabs, burns, mid-stream urine, wound swabs, and sputum, were collected from patients who were brought to Al-Ramadi Hospital, Iraq. All samples were cultured, and the colonies that were obtained were detected using the VITEK® 2 compact. The ability of biofilms to develop was examined using the microtiter plate technique. The bactericidal effectiveness of GTN was estimated by the broth microdilution technique. The presence of *fimA* and *fimC* in S. marcescens isolates was detected using the polymerase chain reaction (PCR) method. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess the effect of GTN on *fimA* and *fimC* gene expression.

Results: The results demonstrated that GTN has no effect on *S. marcescens* growth; while its biofilm was significantly ($P < 0.05$) influenced. Moreover, all *S. marcescens* isolates had *fimA* and *fimC*, and the presence of GTN reduced the expression of these genes.

Conclusion: The findings of this study reveal that GTN can act as a promising antibiofilm agent in reference to S. marcescens.

Keywords: *fimA*, *fimC*, Glyceryl trinitrate, *Serratia marcescens*

Introduction

Serratia marcescens is a gram-negative, opportunistic, and motile nosocomial pathogen of the Enterobacteriaceae family. It produces the characteristic red pigment known as prodigiosin.[1] Fimbria or pili, the synthesis of which is reportedly associated with the development of biofilm in *S. marcescens*, are used by bacteria to adhere to tissues in order to cause infections. The *fimABCD* operon encodes type I pili. The primary fimbrial subunits of *S. marcescens* are known to be *fimA* and *fimC*.[2] The biofilms produced by *S. marcescens* are distinct from those produced by other species of traditional biofilm-forming bacteria, such as *P. aeruginosa* and *E. coli*, which are composed of undifferentiated cells arranged in microcolonies.[3]

According to Labbate *et al.*[4] the development of *S. marcescens* biofilms is a genetically controlled process. This results in cell and structure differentiation, as evidenced by the development of extended threadlike cells, bacterial cell assemblages alongside the threadlike cells, and interconnected cell aggregates as the biofilm matures. These bacteria exhibit both intrinsic and multidrug resistance to many drugs. Treatment success for *S. marcescens* as a nosocomial causative agent depends heavily on the timing of medication depending on the results of antibiotic susceptibility tests.[5] According to a local investigation by Zahraa and Saad,[6] 34 (68%) of the *S. marcescens* isolates produced extended-range beta-lactamases.

Glyceryl trinitrate (GTN) or nitroglycerin (1, 2, 3 propanetriol trinitrate) is an organic nitrate that acts as a vasodilator.[7] Glycerol trinitrate possesses anti-microbial characteristics in addition to its anti-hypertensive effect. It has the potential to suppress *Candida albicans* and *P. aeruginosa* planktonic growth.[8,9] Glycerol trinitrate significantly inhibited biofilm, staphyloxanthin, and oxidative stress tolerance in *S. aureus*.[10]

The present study was aimed at investigating the role of GTN in the biofilm of *S. marcescens,* as a replacement for antibiotic therapy.

Materials and Methods

Isolation and Identification of Bacterial Isolates

From February to June 2021, 316 different specimens, including ear swabs, burns, mid-stream urine, wound swabs, and sputum, were collected from patients who were brought to Al-Ramadi Hospital, Iraq. An informed consent according to the Declaration of Helsinki was obtained from all participants. A sterile cotton swab was used to collect the swabs, and they were then taken to the laboratory in a sterile tube containing normal saline solution. All samples were cultured on blood agar and aerobically incubated at 37°C for 24 hours. The colonies that formed were grown on MacConkey agar and incubated under the same conditions. Using the sophisticated colorimetric approach of VITEK 2 Compact (BioMérieux, France), the dark red colonies (lactose fermenters) were chosen for the identification of *S. marcescens*. A Gram-negative (GN) card was used to identify *S. marcescens*.

Determination of Glyceryl Trinitrate's Minimal Inhibitory Concentration

To determine the minimal inhibitory concentration (MIC) of GTN, a broth microdilution test was used,[11] with progressive twofold concentrations (0.01 to 0.175 mg/ ml) of GTN.

Biofilm Assay

Biofilm formation was quantified according to the method described by.[12] All the isolates were grown in brain heart infusion broth for 18 hours at 37°C. Each isolate was diluted in

tryptic soy broth (TSB), which contains 1% glucose, and pipetted thoroughly. A bacterial isolate suspension was adjusted to the 0.5 McFarland standard. An aliquot of 200 µl of an isolate culture was added to three wells of 96-well polystyrene microplates with a U-shaped bottom. The plate was then covered and incubated for 24 hours at 37°C. Following incubation, the microplates were twice cleaned to get rid of loose bacteria, tapped on paper towels (filter paper) to get rid of any remaining water, and then air-dried. Each well was fixed for 20 minutes at room temperature with 200 µl of absolute methanol. An aliquot of 200 µl of 0.1% crystal violet was applied for 15 minutes. After the staining reaction was completed, excess dye was removed by repeatedly washing (2–3 washes) with distilled water. The plates were then dried by leaving them at room temperature for around 30 minutes to ensure they were completely dry. Following that, 200 µl of 95% ethanol was placed into each well and left there for 10 minutes. Ethanol was used to dissolve the crystal violet dye that was attached to the adherent cells. The experiment was performed in triplicate, with the absorbance of bacteria-free TSB-containing wells representing the negative control. Quantification was carried out using a microplate reader at OD_{600}. The classification of bacterial adherence presented in Table 1 was utilized for data simplification and computation based on OD_{600} values obtained for individual isolates of *S. marcescens*.
The cut-off value (ODc) was determined as follows:

$$ODc = OD_{600} \text{ of the negative control} + 3 \text{ (standard deviation)} \quad (1)$$

All experiments followed the same procedure to assess the effect of GTN on biofilm, with one exception. Tryptic soy broth contained GTN at a concentration of 0.175 mg/ml.

Detection of fimA and fimC Genes using the Polymerase Chain Reaction

The genomic DNA of the bacterial isolates was extracted using the ABIOpure™ Total DNA (USA) kit. PCR reaction tubes containing the mixture were placed in the thermocycler, and DNA was amplified using the primers grouped in Table 2 using the reactants indicated in Table 3. The PCR conditions were optimized by using gradient PCR (Table 4). Thereafter, amplicons were resolved on a 1.5% agarose gel.

Measurement of Gene Expression using Quantitative Real-time Polymerase Chain Reaction

In addition to examining the effect of GTN on target gene expression, a biofilm investigation for five isolates (whose biofilm was strongly influenced by the GTN) was carried out in microtiter plates. A similar technique was used with GTN, including Mueller Hinton broth at 0.175 mg/ml. RNA was extracted from the selected isolates with and without GTN treatment using TRIzol™ reagent following the manufacturer's instructions. The primers listed in Table 2 were used in the real-time polymerase chain reaction (qRT-PCR). Table 5 summarizes the reaction combinations. Also, the final procedure is displayed in Table 4. Relative quantitation was used to determine gene expression levels. The fold changes were assessed between the treated groups and each gene's calibrators.[13] These values were normalized to *rplU* as shown below:

$$\textbf{Folding} = 2^{-\Delta\Delta CT} \quad (1)$$

Table 1. **Calculation of biofilm intensity**

Mean OD_{600}	Biofilm intensity
OD ≤ ODc*	Non-biofilm producer
ODc < OD ≤ 2ODc	Weak
2ODc < OD ≤ 4ODc	Moderate
OD > 4ODc	Strong

*Cut off value
Source: Khayyat *et al.* (2021)

Table 2. **Primers used in this study**

Primer Name		Sequence (5´–3´)	Product size (bp)
fimA	F	ACTACACCCTGCGTTTCGAC	259
	R	GCGTTAGAGTTTGCCTGACC	
fimC	F	AAGATCGCACCGTACAAACC	259
	R	TTTGCACCGCATAGTTCAAG	
rplU	F	GCTTGGAAAAGCTGGACATC	192
	R	TACGGTGGTGTTTACGACGA	

Source: Srinivasan *et al.* (2017)

Table 3. **Components of conventional polymerase chain reaction**

Component	Volume (µl)
Master Mix	10 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Nuclease free water	6 µl
Template DNA	2 µl
Final volume	20 µl

Table 4. **Polymerase chain reaction amplification program**

Step	Temperature (°C)	Minute:Second	Cycles
Initial Denaturation	95	10:00	1
Denaturation	95	00:45	40
Annealing	57	00:45	
Extension	72	00:50	

Source: Srinivasan *et al.* (2017)

Table 5. **Components of the quantitative real-time polymerase chain reaction (qRT-PCR)**

Master mix components	Volume (µl)
qPCR Master Mix	5
RT mix	0.25
$MgCl_2$	0.25
Forward primer	0.5
Reverse primer	0.5
Nuclease Free Water	1.5
RNA	2
Total volume	10

$$\Delta\Delta C_t = \Delta C_{t\ \text{Treated (T)}} - \Delta C_{t\ \text{Untreated (C)}} \quad (2)$$

$$\Delta C_t = C_{t\ \text{of target gene}} - C_{t\ \text{of housekeeping gene}} \quad (3)$$

A fold change of less than twofold was considered insignificant.[14] A melting curve was obtained with temperatures ranging from 72 to 95°C at 0.3°C/s.

Statistical Analysis

Each experiment was replicated three times ($n = 3$). Data were analyzed using GraphPad Prism 9 software using a two-tailed Student's *t*-test and a one-way ANOVA. A *P*-value of < 0.05 was considered significant.

Results

Occurrence of Bacterial Isolates in Specimens

Out of 360 specimens, 305 showed positive bacterial growth on blood agar and MacConkey agar. Using the VITEK 2 compact system, 50 (16.3%) isolates were identified as *S. marcescens*. The highest isolation percentage was from wound infections (42%, $n = 21$), followed by burns (34%, $n = 17$). Values of 10% ($n = 5$) and 14% ($n = 7$) were observed for midstream urine and sputum specimens, respectively. Nevertheless, the bacterium was not isolated from ear swabs.

Biofilm Formation by Serratia marcescens

All *S. marcescens* isolates produced biofilm. However, a one-way ANOVA showed no differences between the biofilms that were produced ($P > 0.05$). In addition, 15 and 35 isolates, respectively, formed weak and moderate biofilms. None of the isolates could develop a strong biofilm. On the other hand, GTN significantly ($P < 0.05$) reduced the biofilm intensity of *S. marcescens* isolates, as shown in Figure 1.

fimA and fimC Detection in Bacterial Isolates

The PCR amplification revealed the presence of *fimA* and *fimC* genes in all the *S. marcescens* isolates, as presented in Figure 2.

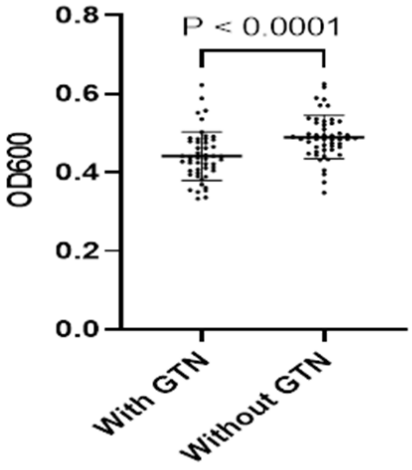

Fig. 1 **Effect of glyceryl trinitrate on *Serratia marcescens* biofilms. Asterisks: Significant difference over the control; Horizontal lines: Mean ± standard deviation.**

Effect of Glyceryl Trinitrate on FimA and FimC Gene Expression

Figure 3 showed that GTN reduced the expression of the *fimA* and *fimC* genes in three isolates, while the gene expression in the other two isolates was unaffected.

Discussion

Serratia marcescens is a significant human opportunistic pathogen that has been linked to a variety of nosocomial infections, including bacteremia, respiratory tract infections, eye infections, and most significantly, urinary tract infections. It produces biofilm and releases a range of virulence factors through a signal-mediated QS mechanism.[15] In Mosul, Iraq, Ali[16] reported that 150 different specimens were isolated, including 3 from blood (15%), 2 from throat swabs (20%), 3 from urine (6%), and 4 from wounds (8%). In Basra, Iraq, Mahdi[17] stated that out of 160 blood samples from neonatal patients in the neonatal intensive care unit, 5 isolates were identified as *S. marcescens*. Regardless of the reservoir, *S. marcescens* has a rather wide distribution among patients with a variety of clinical cases, and hospital staff is thought to be the main source of *S. marcescens* infection spread by direct contact.[18] Patients who have severe clinical problems, long hospital stays, and repeated medical interventions are more likely to contract an infection since these factors require more frequent and intense direct contact with staff hands.[19] The presence of type 1 fimbriae (Figure 2) and the downregulation of the *fimA* and *fimC* genes are evidence that all *S. marcescens* formed biofilm, as can be observed from the current results. In spite of that, GTN had no impact on *S. marcescens*' development.

A search for an alternative therapy option besides antibiotics is essential given the significant antibiotic resistance of *S. marcescens*. The findings of this study revealed that GTN can serve this function because it has no effect on cell viability. Instead, it damaged the pathogen's biofilm, which is an essential component of its pathogenicity.

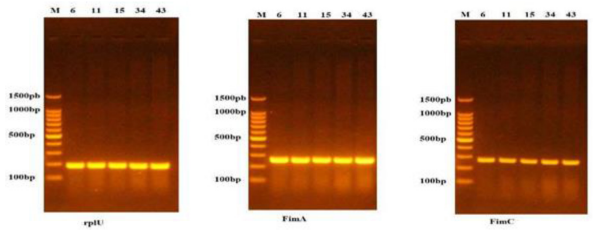

Fig. 2 ***fimA*, *fimC*, and *rplU* amplicons of selected *Serratia marcescens* isolates run on ethidium bromide-containing agarose gel (1.5%) at 5 V/cm.**

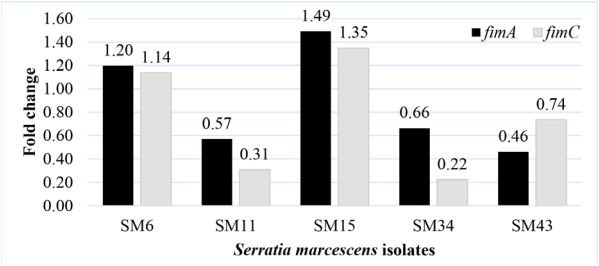

Fig. 3 **Effect of glyceryl trinitrate on the *fimA* and *fimC* expression.**

Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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