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Evaluation of Phenotyping and Genotyping Characterization of *Serratia marcescens* after Biofield Treatment

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Abstract

Serratia marcescens (*S. marcescens*) is Gram-negative bacterium, associated with hospital-acquired infections (HAIs), especially urinary tract and wound infections. The present study was aimed to evaluate the impact of biofield treatment on phenotyping and genotyping characteristics such as antimicrobial susceptibility, biochemical reactions, biotype, DNA polymorphism, and phylogenetic relationship of *S. marcescens* (ATCC 13880). The lyophilized cells of *S. marcescens* were divided into three groups (G1, G2, and G3). Control group (G1) and treated groups (G2 and G3) of *S. marcescens* cells assessed with respect to antimicrobial susceptibility, and biochemical reactions. In addition to that, samples from different groups of *S. marcescens* were evaluated for DNA polymorphism by Random Amplified Polymorphic DNA (RAPD), and 16S rDNA sequencing in order to establish the phylogenetic relationship of *S. marcescens* with different bacterial species. The treated cells of *S. marcescens* showed an alteration of 10.34% and 34.48% antimicrobials in G2 and G3 on 10th day, respectively as compared to control. The significant changes of biochemical reactions were also observed in treated groups of *S. marcescens*. The RAPD data showed an average range of 16-49.2% of polymorphism in treated samples as compared to control. Based on nucleotide homology sequences and phylogenetic analysis, the nearest homolog genus-species was found to be *Pseudomonas fluorescence*. These findings suggest that biofield treatment can prevent the emergence of absolute resistance to the useful antimicrobials against *S. marcescens*.

Keywords: Antimicrobials; Biofield treatment; Polymorphism; Microbial resistance; RAPD; *S. marcescens*

Introduction

Currently, many microorganisms have been acquired the resistance to number of antibiotics and other antimicrobial agents, which were effectively used earlier to cure a microbial infections. The antimicrobial resistant microbes (including bacteria, viruses, fungi, and parasites) can survive in antimicrobial drugs therapy. Therefore, regular treatments are ineffective. The frequent and improper use or misuse of antimicrobial medicines accelerates the emergence of drug-resistant microorganism, which was further spread by meagre infection control and poor sanitary conditions [1]. *Serratia marcescens* (*S. marcescens*) is a rod-shaped Gram-negative bacteria, belongs to family *Enterobacteriaceae*. It is a facultative anaerobic bacterium that can grow in presence and absence of oxygen at temperatures 30°C to 37°C. *S. marcescens* become an opportunist pathogen causing nosocomial infections and commonly involved in hospital-acquired infections (HAIs); specially urinary tract infections (UTIs), pneumonia, septicemia, meningitis and wound infections. Recently, *S. marcescens* drastically acquired the resistance to several existing antimicrobials like penicillin by decreasing the permeability and by β -lactamase to cleave the β -lactam ring of penicillin; fluoroquinolones (nalidixic acid, ciprofloxacin, ofloxacin, and norfloxacin), by proton dependent multidrug resistance (MDR) efflux pumps [2,3]. Therefore, development of effective antimicrobial therapy against *S. marcescens* is very needful for human health. Recently, biofield treatment came in

focus that can cure the microbial infection by changing the microbial susceptibility against the antimicrobial drugs.

The relation between mass-energy was described by Friedrich, then after Einstein gave the well-known equation $E=mc^2$ for light and mass [4,5]. The mass (solid matter) is consist of energy and once this energy vibrates at a certain frequency, it gives physical, atomic and structural properties like shape, size, texture, crystal structure, and atomic weight to the matter. Similarly, human body also consists of vibratory energy particles like neutrons, protons, and electrons. Due to the vibration of these particles in the nucleus, an electrical impulse is generated [6]. Consequently, as per Ampere-Maxwell-Law, varying of these electrical impulses with time generates magnetic field, which cumulatively form electromagnetic field [7,8]. Thus, human has the ability to harness the energy from environment or universe and can transmit into any living or nonliving object(s) around the Globe. The objects always receive the energy and responding into useful way that is called biofield energy and the process is known as biofield treatment. Mr. Mahendra Trivedi's biofield treatment (The Trivedi Effect®) has been applied to transform the structural, physical, and chemical properties of materials in several fields like material science [9-16], agriculture [17-19], and biotechnology [20,21]. The biofield treatment has considerably altered the genotype of the microbes and thereby changed in susceptibility to antimicrobials [22-24].

After consideration of clinical significance of *S. marcescens* and significant impact of biofield treatment on microbes, we felt a detailed investigation was required to evaluate the effect of biofield treatment on *S. marcescens*. After that, the organism was assessed in relation to antimicrobials susceptibility and biotyping based on various

biochemical reactions. We also explored the genotyping of this organism using polymerase chain reaction (PCR) based methodologies of randomly amplified polymorphic DNA (RAPD) and 16S rDNA sequencing techniques. To the best of our knowledge, this is the first report that explores the impact of biofield treatment on *S. marcescens*.

Materials and Methods

Two vials of *S. marcescens* [American Type Culture Collection (ATCC) 13880] were procured from MicroBioLogics, Inc., USA, in sealed packs, and stored as per the recommended storage conditions until further use. The anti-microbial susceptibility, biochemical reactions, and biotype number were evaluated on MicroScan Walk-Away® (Dade Behring Inc., West Sacramento, CA) using Negative Breakpoint Combo 30 (NBPC30). DNA Fingerprinting by RAPD analysis (using Ultrapure Genomic DNA Prep Kit; Cat KT 83) and the 16S rDNA sequencing studies were carried out using Ultrapure Genomic DNA Prep Kit; Cat KT 83 (Bangalore Genei, India). All the tested antimicrobials, biochemicals and other reagents were procured from Sigma-Aldrich.

Study design

The microorganisms were grouped as per study design like bacterial cell were divided in to three groups G1 (control), G2 (treatment, revived), and G3 (treatment, lyophilized). The treatment groups (G1 and G2) were in sealed pack and handed over to Mr. Trivedi for biofield treatment under laboratory condition. Mr. Trivedi provided the treatment through his energy transmission process to the treated groups without touching the samples. After that, G2 group was assessed for antimicrobial susceptibility and biochemical reactions on 5th and 10th day of incubation; and G3 group was assessed on 10th day of treatment. The treated groups were compared with respect to control.

Investigation of antimicrobial susceptibility of *S. marcescens*

Antimicrobial susceptibility of *S. marcescens* was investigated with the help of automated instrument, MicroScan Walk-Away® using Negative Breakpoint Combo 30 (NBPC30) panel as per the manufacturer's instructions [25]. Briefly, after inoculation and rehydration with a standardized suspension of *S. marcescens*, were incubated at 35°C for 16 h. The minimum inhibitory concentration (MIC) and a qualitative susceptibility like susceptible (S), intermediate (I), inducible β -lactamases (IB), and resistant (R) were determined by observing the lowest antimicrobial concentration showing growth inhibition [26]. In the present study, the following 29 antimicrobials were used like amikacin, amoxicillin/k-clavulanate, ampicillin/sulbactam, ampicillin, aztreonam, cefazolin, cefepime, cefotaxime, cefotetan, cefoxitin, ceftazidime, cefuroxime, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gatifloxacin, gentamicin, imipenem, levofloxacin, meropenem, moxifloxacin, nitrofurantoin, norfloxacin, piperacillin, tazobactam, ticarcillin, tobramycin, and vancomycin.

Biochemical studies

The biochemical studies of *S. marcescens* were determined by MicroScan Walk-Away® where, interpretation of biochemical reactions for microbial identification of Gram-negative organisms resulted in high accuracy [27,28]. In this study, the following 31 biochemicals were used like acetamide, adonitol, arabinose, arginine, cetrimide, cephalothin, citrate, colistin, esculin hydrolysis, nitrofurantoin,

glucose, hydrogen sulfide, indole, inositol, kanamycin, lysine, malonate, melibiose, nitrate, oxidation, galactosidase, ornithine, oxidase, raffinose, rhamnose, sorbitol, sucrose, tartrate, tobramycin, urea, and Voges-Proskauer.

Biotype number

The biotype number of *S. marcescens* was determined by MicroScan Walk-Away® processed panel data utilizing biochemical reactions data [25].

Random Amplified Polymorphic DNA (RAPD) analysis

Three inoculums (one for control and other two for treatment named as treatment A and B) were prepared of *S. marcescens* samples. Two inoculums (treatment samples A and B) were subjected to Mr. Trivedi's biofield treatment. After that, the treated samples were sub-cultured by taking 1% inoculum and inoculated to fresh 5 mL medium and labeled as treatment A-1 and treatment B-1, respectively. All samples were incubated at 37°C with 160 rpm for 18 h. Subsequently, the cultures were spun down, and genomic DNA was isolated for control and treated samples using Genomic DNA Prep Kit (Bangalore Genei, India). RAPD was performed with all samples of *S. marcescens* using five RAPD primers, which were labelled as RBA8A, RBA13A, RBA20A, RBA10A and RBA15A. The PCR mixture contained 2.5 μ L each of buffer, 4.0 mM each of dNTP, 2.5 μ M each of primer, 5.0 μ L each of genomic DNA, 2 U each of Taq polymerase, 1.5 μ L of MgCl₂ and 9.5 μ L of water in a total of 25 μ L with the following PCR amplification protocol; initial denaturation at 94°C for 7 min, followed by 8 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, and extension at 72°C for 2 min; and 35 cycle of denaturation at 94°C for 1 min, annealing at 38°C for 1 min, and extension at 72°C for 1.5 min; and the final extension at 72°C for 7 min. Amplified PCR products from all samples (control and treated) were separated on 1.5 % agarose gels at 75 volts, stained with ethidium bromide and visualized under UV illumination.

Amplification and gene sequencing of 16S rDNA

Genomic DNA was isolated from *S. marcescens* cells by using genomic purification Kit, according to the instructions of manufacturer. 16S rDNA gene (~1.5 kb) was amplified by universal primers; forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (3'-ACGGTCATACCTTGTACGACTT-5'). Amplified products were subjected to electrophoresis in 1.0% agarose gel, stained with ethidium bromide and visualized under UV light in a gel documentation unit (BioRad Laboratories, USA). The PCR amplified fragment was purified from the agarose gel using a DNA Gel Extraction Kit. Sequencing of amplified product was done on commercial basis from Bangalore Genei, India. The 16S rDNA sequences obtained were aligned and compared with the sequences stored in Gene Bank data base available from National Center for Biotechnology Information (NCBI) using the algorithm BLASTn program. Multiple sequence alignment/phylogenetic tree were established using MEGA3.1 molecular software [29].

Results

Assessment of antimicrobial susceptibility

The effect of biofield treatment on *S. marcescens* to susceptibility pattern and MIC of selected antimicrobials are summarized in Tables 1

and 2, respectively. The data were analyzed and compared with respect to control. The treated cells of *S. marcescens* showed an alteration of 10.34% and 34.48% in G2 and G3 group on 10th day, respectively of antimicrobials susceptibility among all tested antimicrobials as compared to control. Studying the effect of biofield treatment in the antibiogram of *S. marcescens*, revealed that the amikacin and tobramycin were converted from resistance to susceptible on 10th day of G3 group as compared to control. Aztreonam, cefotetan, ceftazidime, cefuroxime and chloramphenicol were converted from resistance to intermediate on 10th day of biofield treatment of G3 group as compared to control. The cefepime and cefotaxime were converted from resistance to intermediate on 10th day of G2 treated cells and complete susceptibility was observed for gentamicin and cefepime on 10th day of G3 treated cells as compared to control (Table 1). It was also observed that there was reduced activity of inducible β -lactamase of aztreonam, cefotaxime, cefotetan, ceftazidime, and ceftriaxone antimicrobials. The MIC values of amikacin, aztreonam, cefepime, cefotetan, ceftazidime, gentamicin and tobramycin were decreased about two-folds; whereas about four-folds decrease in MIC values of cefotaxime and ceftriaxone on 10th day of G2 treated cells as compared to control (Table 2).

S. No.	Antimicrobial	Control	G2		G3
		G1	5 th day	10 th day	10 th day
1	Amikacin	R	R	R	S
2	Amoxicillin/K-clavulanate	R	R	R	R
3	Ampicillin/Sulbactam	R	R	R	R
4	Ampicillin	R	R	R	R
5	Aztreonam	R	R	R	IB
6	Cefazolin	R	R	R	R
7	Cefepime	R	R	I	S
8	Cefotaxime	R	R	I	IB
9	Cefotetan	R	R	R	IB
10	Cefoxitin	R	R	R	R
11	Ceftazidime	R	R	R	IB
12	Cefuroxime	R	R	R	R
13	Ceftriaxone	I	I	IB	IB
14	Cephalothin	R	R	R	R
15	Chloramphenicol	R	R	R	I
16	Ciprofloxacin	S	S	S	S
17	Gatifloxacin	S	S	S	S
18	Gentamicin	R	R	R	S
19	Imipenem	S	S	S	S
20	Levofloxacin	S	S	S	S
21	Meropenem	S	S	S	S

22	Moxifloxacin	S	S	S	S
23	Nitrofurantoin	R	R	R	R
24	Norfloxacin	S	S	S	S
25	Piperacillin	IB	IB	IB	IB
26	Tazobactam	IB	IB	IB	IB
27	Ticarcillin	IB	IB	IB	IB
28	Tobramycin	R	R	R	S
29	Vancomycin	S	S	S	S

G stands for group; I: intermediate; S: susceptible; R: resistant; IB: inducible β -lactamase.

Table 1: Effect of biofield treatment on *S. marcescens* to susceptibility pattern of selected antimicrobials.

S. No.	Antimicrobial	Control	G2		G3
		G1	5 th day	10 th day	10 th day
1	Amikacin	>32	>32	>32	≤16
2	Amoxicillin/K-clavulanate	≥16/8	≥16/8	≥16/8	≥16/8
3	Ampicillin/Sulbactam	≥16/8	≥16/8	≥16/8	≥16/8
4	Ampicillin	≥16	≥16	≥16	≥16
5	Aztreonam	>16	>16	>16	≤8
6	Cefazolin	≥16	≥16	≥16	≥16
7	Cefepime	>16	>16	16	≤8
8	Cefotaxime	>32	>32	32	≤8
9	Cefotetan	>32	>32	>32	≤16
10	Cefoxitin	≥16	≥16	≥16	≥16
11	Ceftazidime	>16	>16	>16	≤8
12	Cefuroxime	>16	>16	>16	>16
13	Ceftriaxone	32	32	≤8	≤8
14	Cephalothin	≥16	≥16	≥16	≥16
15	Chloramphenicol	>16	>16	>16	16
16	Ciprofloxacin	≤1	≤1	≤1	≤1
17	Gatifloxacin	≤2	≤2	≤2	≤2
18	Gentamicin	>8	>8	>8	≤4
19	Imipenem	≤4	≤4	≤4	≤4
20	Levofloxacin	≤2	≤2	≤2	≤2
21	Meropenem	≤4	≤4	≤4	≤4
22	Moxifloxacin	≤2	≤2	≤2	≤2
23	Nitrofurantoin	≥64	≥64	≥64	≥64

24	Norfloxacin	≤4	≤4	≤4	≤4
25	Piperacillin	≤16	≤16	≤16	≤16
26	Tazobactam	≤16	≤16	≤16	≤16
27	Ticarcillin	≤16	≤16	≤16	≤16
28	Tobramycin	>8	>8	>8	≤4
29	Vancomycin	≤2	≤2	≤2	≤2
G stands for group; MIC data are presented in µg/mL.					

Table 2: Effect of biofield treatment on *S. marcescens* to MIC of selected antimicrobials.

Organism identification by biochemical reactions

The biochemical reactions of *S. marcescens* are presented in Table 3. In the present study, acetamide, cetrinide, indole, inositol, and oxidase biochemical reactions of control and treated cells of *S. marcescens* showed negative biochemical reactions.

S. No.	Code	Biochemical	Control	G2		G3
			G1	5 th day	10 th day	10 th day
1	ACE	Acetamide	-	-	-	-
2	ADO	Adonitol	+	+	+	+
3	ARA	Arabinose	+	+	+	-
4	ARG	Arginine	+	+	-	-
5	CET	Cetrinide	-	-	-	-
6	CF8	Cephalothin	+	+	+	+
7	CIT	Citrate	+	+	+	+
8	CL4	Colistin	+	+	+	+
9	ESC	Esculin hydrolysis	+	+	+	+
10	FD64	Nitrofurantoin	+	+	+	+
11	GLU	Glucose	+	+	+	+
12	H2S	Hydrogen sulfide	+	+	+	-
13	IND	Indole	-	-	-	-
14	INO	Inositol	-	-	-	-
15	K4	Kanamycin	+	+	+	-
16	LYS	Lysine	+	+	+	+
17	MAL	Malonate	+	+	+	-
18	MEL	Melibiose	+	+	+	-
19	NIT	Nitrate	+	+	+	+
20	OF/G	Oxidation	+	+	+	+

21	ONPG	Galactosidase	+	+	+	+
22	ORN	Ornithine	+	+	+	+
23	OXI	Oxidase	-	-	-	-
24	RAF	Raffinose	+	+	+	-
25	RHA	Rhamnose	+	+	+	-
26	SOR	Sorbitol	+	+	+	+
27	SUC	Sucrose	+	+	+	+
28	TAR	Tartrate	-	+	+	-
29	TO4	Tobramycin	+	+	+	-
30	URE	Urea	+	+	+	-
31	VP	Voges-Proskauer	+	+	+	+
G stands for group; - (negative); + (positive).						

Table 3: Effect of biofield treatment on *S. marcescens* to biochemical reactions.

Twenty-four of thirty-one biochemical reactions were showed positive reaction for control and two treatment groups. Arginine reaction of treated G2 cells on 10th day was negative and tartrate reaction was positive for the treatment G2 cells on both 5th and 10th day as compared to control. Ten out of thirty one biochemical reactions (32.25 %) of treated cells in G3 were converted from positive to negative reaction, and tartrate biochemical reaction was remain unchanged as negative as compared to control (Table 3).

Organism identification by biotype number

The biotype number of *S. marcescens* was determined by MicroScan Walk-Away® processed panel, using biochemical reactions data. There was no change in biotype number observed in treated G2 cells on 5th day of incubation. However, the significant changes in the biotype number of *S. marcescens* were observed in G2 and G3 on 10th day of incubation as compared to control (Table 4).

Feature	Control	G2		G3
	G1	5 th day	10 th day	10 th day
Biotype Number	7736 7376	7736 7376	7736 5376	7020 5356
Organism Identification Name	<i>S. marcescens</i>	<i>S. marcescens</i>	<i>S. marcescens</i>	<i>S. marcescens</i>
G stands for group.				

Table 4: Effect of biofield treatment on *S. marcescens* to biotype number.

Random Amplified Polymorphic DNA (RAPD) analysis

The DNA polymorphic photograph is shown in Figure 1, and the polymorphic bands are marked by arrows.

The percentage of polymorphism was calculated using following equation:

$$\text{Percent polymorphism} = A/B \times 100;$$

Where, A=number of polymorphic bands in treated sample; and B=number of polymorphic bands in control.

The results of DNA polymorphic patterns are shown in Tables 5 and 6. The level of polymorphism was found about an average range of 16-49.2% of polymorphism in treated samples as compared to control in *S. marcescens*.

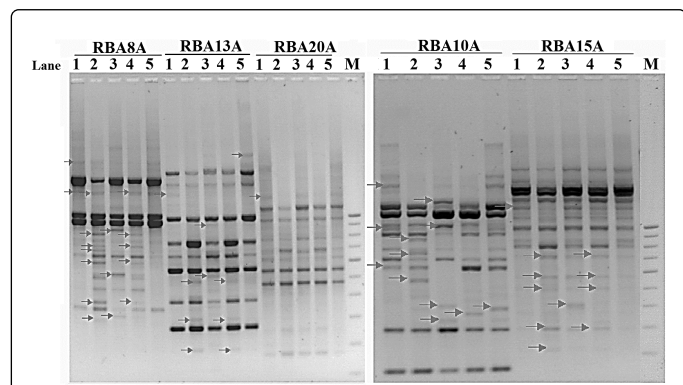


Figure 1: Random amplified polymorphic-DNA fragment patterns of *S. marcescens* generated using five RAPD primers, RBA 8A, RBA 13A, RBA 20A, RBA 10A and RBA 15A. 1, Control; 2, Treated A; 3, Treated A-1; 4, Treated B; 5, Treated B-1; M: 100 bp DNA Ladder.

S. No.	Primer	Nucleotide sequence (5'-3')	Band scores	Common bands in control and treated	Unique band				
					Control	TSA	TSA-1	TSB	TSB-1
1	RBA 8A	GTTTC GCTCC	17	5	2	4	3	3	0
2	RBA 13A	GTGGA TCCGA	14	8	1	2	1	1	1
3	RBA 20A	GCGAT CCCCA	8	7	1	0	0	0	0
4	RBA 10A	CCGCA GCCAA	17	5	1	3	3	2	3
5	RBA 15A	AAGAG CCCGT	15	9	1	2	1	2	0

TSA: treated sample A; TSA-1: treated sample A-1; TSB: treated sample B; TSB-1: treated sample B-1.

Table 5: DNA polymorphism analyzed by random amplified polymorphic DNA (RAPD) analysis.

Primer	C and TSA	C and TSA-1	C and TSB	C and TSB-1	TSA and TSA-1	TSB and TSB-1	TSA and TSB	TSA-1 and TSB-1
RBA 8A	90%	50%	70%	20%	66%	38%	20%	30%

RBA 13A	40%	30%	40%	20%	45%	40%	0.0%	10%
RBA 20A	10%	0.0%	10%	0.0%	41%	10%	0.0%	0.0%
RBA 10A	46%	53%	30%	30%	58%	44%	16%	23%
RBA 15A	60%	20%	50%	10%	50%	28%	10%	10%
Average polymorphism	49.2%	30.6%	40%	16%	52%	32%	9.2%	14.6%

C: control; TSA: treated sample A; TSA-1: treated sample A-1; TSB: treated sample B; TSB-1: treated sample B-1

Table 6: Level of polymorphism between control and treated samples.

16S rDNA genotyping

The 16S rDNA sequence was determined in *S. marcescens*. The alignment and comparison of the gene sequences were performed with the sequences stored in Gene Bank database available from NCBI using the algorithm BLASTn program. The nearest homolog genus-species of *S. marcescens* was found to be *P. fluorescens* (Accession No. DQ439976). Some other close homologs of *S. marcescens* were can be found from the alignment as shown in Table 7.

Alignment view	ID	Alignment result	Sequence description
	8A	0.96	Sample studied
	EU233 275	0.96	<i>Serratia marcescens</i> strain RJT
	AB061 685	0.98	<i>Serratia marcescens</i>
	EF208 030	0.97	<i>Serratia marcescens</i> strain A3
	EF194 094	0.97	<i>Serratia marcescens</i> strain H3010
	DQ439 976	0.98	<i>Pseudomonas fluorescens</i> strain ost5
	AB091 837	0.98	<i>Pseudomonas fluorescens</i>
	EU036 987	0.97	<i>Serratia nematodiphila</i> strain DZ0503SBS1
	EF627 046	0.97	<i>Serratia marcescens</i> strain cocoon-1
	AJ233 431	1	<i>Serratia marcescens</i> (strain DSM 30121)
	DQ417 332	0.96	<i>Serratia marcescens</i> strain 6CW

Table 7: The closest sequences of *S. marcescens* from sequence alignment using NCBI GenBank and ribosomal database project (RDP).

The distance matrix based on nucleotide sequence homology data are presented in Figure 2. Based on nucleotides homology and phylogenetic analysis the microbe (Sample 8A) was detected to be *S. marcescens* (GenBank Accession Number: EU233275). Phylogenetic

enzymatic level after biofield treatment. Therefore, biofield treatment could be applied to improve the sensitivity of antimicrobials against microbial resistance.

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