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Bacterial Identification Using 16S rDNA Gene Sequencing and Antibiogram Analysis on Biofield Treated *Pseudomonas fluorescens*

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Abstract

Biofield therapies have been reported to improve the quality of life as compared to other energy medicine. The aim of the study was to evaluate the impact of Mr. Trivedi's biofield energy treatment on *Pseudomonas fluorescens* (*P. fluorescens*) for antimicrobial sensitivity, minimum inhibitory concentration (MIC), biochemical reactions, and biotype number. *P. fluorescens* cells were procured from MicroBioLogics Inc., USA in sealed packs bearing the American Type Culture Collection (ATCC 49838) number and divided in control and treated group. The effect was evaluated on day 10, and 159 after biofield treatment in lyophilized state. Further study was performed on day 5, 10, and 15 after retreatment on day 159 in revived state as per study design. All experimental parameters were studied using automated MicroScan Walk-Away® system. The 16S rDNA sequencing was carried out to correlate the phylogenetic relationship of *P. fluorescens* with other bacterial species after treatment. The results showed improved sensitivities and decreased MIC value of aztreonam, cefepime, moxifloxacin, and tetracycline in revived and lyophilized treated sample with respect to the control. Arginine, cetrimide, kanamycin, and glucose showed altered biochemical reactions after biofield treatment with respect to control. Biotype numbers were altered along with species in lyophilized as well as in revived group. Based on nucleotides homology and phylogenetic analysis using 16S rDNA gene sequencing, treated sample was detected to be *Pseudomonas entomophila* (GenBank Accession Number: AY907566) with 96% identity of gene sequencing data, which was nearest homolog species to *P. fluorescens* (Accession No. EF672049). These findings suggest that Mr. Trivedi's unique biofield treatment has the capability to alter changes in pathogenic *P. fluorescens* even in the lyophilized storage condition and can be used to modify the sensitivity of microbes against antimicrobials.

Keywords: *Pseudomonas fluorescens*; Biofield treatment; Lyophilized state; Antimicrobial susceptibility; Biochemical reactions; Biotype; 16S rDNA sequencing

Abbreviations

NCCAM: National Center for Complementary and Alternative Medicine; ATCC: American Type Culture Collection; DNA: Deoxyribonucleic acid; ESBL: Extended spectrum beta-lactamase; MIC: Minimum inhibitory concentration; MEGA: Molecular evolutionary genetics analysis; NBPC30: Negative breakpoint combo panel 30; NCBI: National center for biotechnology information; OTUs: Operational Taxonomic Units; PCR: Polymerase chain reaction; RDP: Ribosomal database project

Introduction

The genus Pseudomonas is a group of ubiquitous Gram-negative rod shaped bacterium mostly present in soil, and water surfaces. *Pseudomonas* infections are related with high morbidity and mortality [1], and common species isolated from clinical specimen *is Pseudomonas aeruginosa* [2]. However, *Pseudomonas fluorescens* (*P. fluorescens*) is a member of the fluorescent pseudomonas group and mostly regarded to be of low virulence and an infrequent human infection [3]. *P. fluorescens* is a heterogeneous species that can be subdivided by various taxonomic criteria into several biotypes [4]. Complete genome sequence was reported in two strains, namely *P. fluorescens* Pf-5 genome consist of 87 RNAs and 6137 proteins, and *P. fluorescens* PfO-1 genome consist of 95 RNAs and 5736 proteins [5,6].

Lyophilization or freeze drying is the most widely used storage methods for drying bacteria or other biological materials. It may be considered as a two-stage process of freezing and drying. Freezing has been extensively used in case of cells and tissues [7], which can be convenient for the survival of microorganisms. Even sensitive microorganism showed satisfactory recoveries, if proper care must be taken with respect to temperature control, suspending media, and rate of cooling [8]. On the other hand, drying is also more advisable storage condition for sensitive microorganism. Above methods involved removal of moisture from a frozen solution or suspension in a high vacuum, but few exceptions in case of nonviable materials, especially proteins and tissues. This approach has been extensively used for different substance in past 40 years by numerous investigators [9,10]. Although, alterations in microbes cannot be happened in lyophilized state without any strong energy transmission. In order to evaluate the impact of Mr. Trivedi's biofield treatment on lyophilized strain, study was designed to investigate the alteration in antibiogram pattern and its related parameters. Mr. Trivedi has the ability to harness the energy from the environment or Universe and transmit this energy into any object (living or nonliving) on the Globe. The objects always receive the energy and responding into useful way that is knows biofield energy. This process is termed as biofield treatment. Mr. Trivedi's unique biofield treatment is also known as The Trivedi Effect®. Biofield, the electromagnetic field that surrounds the living organism will provides regulatory and communication functions within the organism. Mr. Trivedi's biofield energy treatment was extensively studied in material

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science [11-13], agricultural science [14-16], in biotechnology [17]. It is reported in altering the susceptibility of antimicrobials of pathogenic microbes [18-20] and reduced the viral load in hepatitis C virus [21].

After consideration of clinical significance of *P. fluorescens* and significant impact of biofield treatment on microbes, a detailed investigation was studied to evaluate the impact of biofield treatment on *P. fluorescens*. Treatment was assessed in relation to antimicrobials susceptibility, biotyping based on various biochemical reactions. Further, genotyping of this organism was performed using 16S rDNA sequencing method.

Materials and Methods

Lyophilized sample of *Pseudomonas fluorescens* [American Type Culture Collection (ATCC) 49838] was procured from MicroBioLogics, Inc., USA, and stored as per suggested storage conditions until further use. The acceptability of the identification media and antimicrobial agents were checked prior to the study by ATCC organisms. The antimicrobial susceptibility, biochemical reactions, and biotype number were evaluated on MicroScan Walk-Away® (Dade Behring Inc., West Sacramento, CA) using Negative Breakpoint Combo 30 (NBPC30) panel. The NBPC30 panel was stored at 2 to -25°C. The panels were allowed to equilibrate to room temperature prior to rehydration. All opened panels were used in same day. The 16S rDNA sequencing study was carried out using Ultrapure Genomic DNA Prep Kit; Cat KT 83 (Bangalore Genei, India).

Inoculum preparation

The turbidity standard technique using direct inoculation of revived and lyophilized *P. fluorescens* was used. Using a sterile wooden applicator stick or bacteriological loop, the surface of 4-5 large or 5-10 small morphologically similar culture was touched for well-isolated colonies from an 18-24 hour non-inhibitory agar plate. Further, colonies were emulsified in 3 mL of inoculum water (autoclaved deionized water) to an equivalent of a 0.5 McFarland barium sulfate turbidity standard. 100 µL of the standardized suspension was pipetted into 25 mL of inoculum water using pluronic and inverted 8-10 times.

Experimental design

Experimental design for lyophilized sample of *P. fluorescens* was divided into two main groups (Gr.) namely- Gr I and Gr. II

Group I: No treatment was given. Revived from lyophilized state and considered as control. It was analyzed for antimicrobial sensitivity, minimum inhibitory concentration (MIC), biochemical reactions and biotype number as per the standard protocol.

Group II: This group was divided into two separate sub parts named as Gr. IIA and Gr. IIB.

Group IIA: *P. fluorescens* sample was subjected to the Mr. Trivedi's biofield treatment for the first time in the lyophilized state itself and then revived. After treatment, the analysis was done on day 10 for antimicrobial sensitivity, MIC, biochemical reactions and biotype number as per the standard protocols. Further, 16S rDNA sequencing study was carried out in treated sample to study the genotypic alteration in organism.

Group IIB: It included the sample which was analyzed in Gr. IIA. *P. fluorescens* strain was stored for 159 days at -70°C, so that no contamination would take place till they were revived again. Gr. IIB was further subdivided in two groups named as Gr. IIB, study I and Gr. IIB, study II.

Group IIB-Study I: After 159 days, antimicrobial sensitivity, MIC, biochemical reactions and biotyping were performed as per the standard protocol.

Group IIB-Study II: The stored strain was revived from -70°C and the revived culture was again subjected to Mr. Trivedi's biofield energy treatment (re-treatment) on day 159. After biofield retreatment, the sample was sub-cultured into three separate tubes on 3 different days (Day 0, Day 5 and Day 10) and was analyzed keeping the main treated tube aside. Each sample was analyzed after 5 days of its sub-culturing.

Biofield treatment modalities

The lyophilized (Gr. IIA) sample of *P. fluorescens* was subjected to biofield treatment followed by retreatment after storing for 159 days in revived state (Gr. IIB, study II). The treatment group in sealed pack was 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. Treated samples were assessed for antimicrobial sensitivity, biochemical reactions, and biotyping as per experimental design. Whilst handing over these cultures to Mr. Trivedi for retreatment purposes, optimum precautions were taken to avoid contamination. The 16S rDNA gene sequencing of *P. fluorescens* was also carried out to confirm the identity of sample after biofield treatment [19].

Antimicrobial sensitivity assay

Antimicrobial sensitivity pattern of *P. fluorescens* in each group was carried out with the help of automated instrument, MicroScan Walk-Away® using NBPC30 panel, as per the manufacturer's instructions [22]. MIC and the qualitative susceptibility pattern like resistant (R), intermediate (I), or susceptible (S), were determined by observing the lowest antimicrobial concentration showing growth inhibition. All these antimicrobials used in this study were purchased from Sigma-Aldrich, USA.

Biochemical studies

The biochemical studies of *P. fluorescens* were performed on MicroScan Walk-Away® [22]. Biochemical reactions pattern were carried out in all the tested group using 33 biochemicals viz. acetamide, adonitol, arabinose, arginine, cetrimide, cephalothin, citrate, colistin, esculin hydrolysis, nitrofurantoin, glucose, hydrogen sulfide, indole, inositol, kanamycin, lysine, malonate, melibiose, nitrate, oxidation-fermentation/glucose, galactosidase, ornithine, oxidase, penicillin, raffinose, rhamnose, sorbitol, sucrose, tartrate, tryptophan deaminase, tobramycin, urea, and Voges-Proskauer. All these biochemicals used in this experiment were procured from Sigma-Aldrich, USA.

Biotype number

The biotype number of *P. fluorescens* was determined by automated MicroScan Walk-Away® processed panel data utilizing biochemical reactions [22].

PCR amplification and gene sequencing of 16S rDNA

Genomic DNA was isolated and purified from treated group of *P. fluorescens* cells by using genomic purification Kit, as per the manufacturer's instructions. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. DNA 16S region amplification was performed using the primer set 16SF–16SR [23]. The 16S rDNA gene (~1.5 kb) was amplified employing universal primers (16SF 5´-AGAGTTTGATCCTGGCTCAG-3´; 16SR 5´-CTACGGCTACCTTGTTACGA-3´). Amplification was carried out

in a Rapid Cycler thermo controller, with initial denaturation, annealing and extension temperature. Following amplification, products were analyzed by gel electrophoresis at 100 V (in 1.0% agarose gel, 0.2 µg of ethidium bromide mL^{-1}) in tris-acetate buffer (TAE), and visualized under UV light in a gel documentation unit (BioRad Laboratories, USA). The amplified fragment of PCR was purified from the agarose gel by DNA Gel Extraction Kit. Sequencing of amplified product was carried out on commercial basis from Bangalore Genei, India. The obtained 16S rDNA sequences data were aligned and compared with the sequences, available in GenBank database of National Center for Biotechnology Information (NCBI) using the algorithm BLASTn program. The multiple sequence alignment/phylogenetic tree were constructed using MEGA 3.1 software using neighbor joining method [24].

Results

Evaluation of antimicrobial susceptibility

The results of antimicrobial sensitivity after biofield treatment on *P. fluorescens* are summarized in Table 1. Biofield treated cells of *P. fluorescens* showed alteration in sensitivity pattern of aztreonam, cefepime, Cefotaxime, ciprofloxacin, Piperacillin, and tetracycline. Biofield treatment in lyophilized state of *P. fluorescens* showed improved sensitivity in case of tetracycline i.e., from intermediate to susceptible in Gr. IIA, on day 10 as compared to its control. Rest of the tested antimicrobials did not show any alteration in Gr. IIA, on day 10 with respect of control.

All the antimicrobials were again tested on Gr. IIB, study I sample and results showed alteration in two antimicrobials i.e., Cefepime and Cefotaxime. Cefepime showed improved sensitivity i.e., from intermediate to susceptible, while sensitivity of Cefotaxime was changed from intermediate to resistant (Gr. IIB, study I) as compared to control (Gr. I). only tetracycline showed changed sensitivity (i.e., S→I) in Gr. IIB, study I (day 159) as compared to analysis done on day 10 (Gr. IIA). However, results showed that most of the tested antimicrobials (except cefepime and Cefotaxime) showed sustained sensitivity profile on storage of treated *P. fluorescens* strain for 159 days as compared to control.

Further study II (Gr. IIB) results showed alteration in sensitivity pattern of tested antimicrobials after biofield retreatment in revived state of *P. fluorescens*, and compared with control. Results showed that sensitivity was improved in case of aztreonam and Cefotaxime antibiotics i.e., from resistant to intermediate on day 10 (Gr. IIB, study II) after retreatment compared with Gr. IIB, study I. Cefotaxime showed altered sensitivity pattern from intermediate to resistant on day 5 and 15 (Gr. IIB, study II) as compared to control. Ciprofloxacin showed altered sensitivity from susceptible to intermediate on day 15 (Gr. IIB, study II) after retreatment as compared to control. Piperacillin sensitivity was also altered from susceptible to intermediate on day 5 and day 15 (Gr. IIB, study II) with respect to control. Tetracycline sensitivity was improved i.e., from intermediate to susceptible on day 10 (Gr. IIB, study II) as compared to control (Gr. I). Rest of antimicrobials such as Amikacin, Ceftazidime, ceftriaxone, chloramphenicol, gentamicin, Imipenem, levofloxacin, Meropenem, Piperacillin/tazobactam, Ticarcillin/k-clavulanate, tobramycin, and trimethoprim/sulfamethoxazole did not show any alteration after treatment in lyophilized or revived state with respect to control.

Determination of minimum inhibitory concentration (MIC)

Biofield treatment in lyophilized and retreatment in revived *P. fluorescens* showed alteration in MIC values of antimicrobials with respect to control. MIC values are summarized in Table 2. Tetracycline showed two-folds decrease in MIC (8 µg/mL to ≤ 4 µg/mL) value in Gr. IIA, day 10, i.e., after biofield treatment in lyophilized state with respect to control (Gr. I). To study the sustained effect of biofield treatment on MIC values, analysis was done after 159 days (Gr. IIB, study I). Results showed that antimicrobials such as cefepime showed two-folds decreased MIC i.e., 16 µg/mL to ≤ 8 µg/mL. Beside, altered MIC value was also reported in ESBL-a Scrn (>4 µg/mL to ≤ 4 µg/mL), ESBL-b Scrn (>1 µg/mL to ≤ 1 µg/mL), Cefotaxime (32 µg/mL to >32 µg/mL), and Norfloxacin (≤ 4 µg/mL to 8 µg/mL) as compared to control (Gr. I).

Gr. IIB, study II, results showed altered MIC values in ten antimicrobials out of thirty-two tested. Decreased value of MIC was reported in case of aztreonam (>16 µg/mL to 16 µg/mL), while two-folds decreased MIC in moxifloxacin (4 µg/mL to ≤ 2 µg/mL), tetracycline (8 µg/mL to ≤ 4 µg/mL) on day 10 (Gr. IIB, study II) as compared to control (Gr. I). Cefotaxime showed altered MIC (32 µg/mL to >32 µg/mL) on day 5 and 15, as compared to control (Gr. I). Norfloxacin (≤ 4 µg/mL to 8 µg/mL) showed two-folds decreased MIC on day 5 and 10, as compared to Gr. IIB, study I. Ciprofloxacin, Gatifloxacin and Norfloxacin showed increased MIC values on day 15 (Gr. IIB, study II) compared to control, while MIC value of moxifloxacin and Piperacillin was altered on day 5, and 15 as compared to control. Rest of the antimicrobials did not show any alteration in MIC value, after treatment in any group with respect to control.

Biochemical reaction study

The biochemical reactions of *P. fluorescens* are reported in Table 3. After biofield treatment, 12.12% alteration in biochemical reactions was reported. Arginine and cetrimide showed negative reactions (i.e., positive (+) to negative (-)), while glucose showed positive reaction (i.e., negative (-) to positive (+)) in Gr. IIA, day 10 as compared to control (Gr. I).

Further, to check the sustained effect on tested biochemicals, analysis was performed on day 159, and the results showed alteration in cetrimide i.e., again positive reaction on day 159 (Gr. IIB, study I), as compared to results of day 10 (Gr. IIA). However, cetrimide and kanamycin showed negative reaction after retreatment in revived state on day 5, 10, and 15 (Gr. IIB, study II) as compared to control. Arginine showed negative reaction on day 5, while glucose showed positive reaction on day 5 and 15 as compared to control (Gr. I). Rest of the twenty-nine biochemical did not show any alteration in reaction pattern with respect to control.

Biotype number study

The biotype number of *P. fluorescens* was determined on MicroScan Walk-Away® processed panel, using biochemical reactions data. Biotype numbers with organism identification are summarized in Table 4. The result showed an alteration in biotype number (4000 0043) in Gr. IIA, day 10 and in Gr. IIB, studies II, day 10 with identified organism as *Vibrio fluvialis* as compared to control. Further results showed an alteration of biotype numbers 4000 0043/0004 0022, with altered species as *Vibrio fluvialis*/*Pseudomonas* spp., as compared to control. After retreatment results showed alteration in biotype number on day 5, 10 and 15 in Gr. IIB, study II as compared to control. Biofield treatment showed altered biotype numbers i.e., 4000 0043, 0204 0622, and 4000 2043 observed on day 5, 10, and 15 respectively in Gr. IIB, study II with respect to control. Altered microorganism was found on day 15 as *Vibrio fluvialis* with respect to control (Gr. I).

S No	Antimicrobial	Gr. I	Gr. IIA	Gr. IIB, Study I	Gr. IIB, Study II		
		Control	Day 10	Day 159	Day +5	Day +10	Day +15
1.	Amikacin	S	S	S	S	S	S
2.	Aztreonam	R	R	R	R	I	R
3.	Cefepime	I	I	S	S	S	S
4.	Cefotaxime	I	I	R	R	I	R
5.	Ceftazidime	S	S	S	S	S	S
6.	Ceftriaxone	I	I	I	I	I	I
7.	Chloramphenicol	R	R	R	R	R	R
8.	Ciprofloxacin	S	S	S	S	S	I
9.	Gentamicin	S	S	S	S	S	S
10.	Imipenem	S	S	S	S	S	S
11.	Levofloxacin	S	S	S	S	S	S
12.	Meropenem	S	S	S	S	S	S
13.	Piperacillin/tazobactam	S	S	S	S	S	S
14.	Piperacillin	S	S	S	I	S	I
15.	Tetracycline	I	S	I	I	S	I
16.	Ticarcillin/k-clavulanate	R	R	R	R	R	R
17.	Tobramycin	S	S	S	S	S	S
18.	Trimethoprim/sulfamethoxazole	R	R	R	R	R	R

Table 1: Effect of biofield treatment on antimicrobial susceptibility of *Pseudomonas fluorescens*. R: Resistant; I: Intermediate; S: Susceptible; Gr.: Group; Antimicrobial susceptibility pattern in control and treated groups were evaluated using automated Micro Scan Walk-Away® system using NBPC30 panel.

S. No.	Antimicrobial	Gr. I	Gr. IIA	Gr. IIB, Study I	Gr. IIB, Study II		
		Control	Day 10	Day 159	Day +5	Day +10	Day +15
1.	Amikacin	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16
2.	Amoxicillin/k-clavulanate	>16/8	>16/8	>16/8	>16/8	>16/8	>16/8
3.	Ampicillin/sulbactam	>16/8	>16/8	>16/8	>16/8	>16/8	>16/8
4.	Ampicillin	>16	>16	>16	>16	>16	>16
5.	Aztreonam	>16	>16	>16	>16	16	>16
6.	Cefazolin	>16	>16	>16	>16	>16	>16
7.	Cefepime	16	16	≤ 8	≤ 8	≤ 8	≤ 8
8.	Cefotaxime	32	32	>32	>32	32	>32
9.	Cefotetan	>32	>32	>32	>32	>32	>32
10.	Cefoxitin	>16	>16	>16	>16	>16	>16
11.	Ceftazidime	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8
12.	Ceftriaxone	32	32	32	32	32	32
13.	Cefuroxime	>16	>16	>16	>16	>16	>16
14.	Cephalothin	>16	>16	>16	>16	>16	>16
15.	Chloramphenicol	>16	>16	>16	>16	>16	>16
16.	Ciprofloxacin	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	2
17.	ESBL-a Scrn	>4	>4	≤ 4	>4	>4	>4

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18.	ESBL-b Scrn	>1	>1	≤ 1	>1	>1	>1
19.	Gatifloxacin	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	4
20.	Gentamicin	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
21.	Imipenem	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
22.	Levofloxacin	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2
23.	Meropenem	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
24.	Moxifloxacin	4	4	4	>4	≤ 2	>4
25.	Nitrofurantoin	>64	>64	>64	>64	>64	>64
26.	Norfloxacin	≤ 4	≤ 4	8	≤ 4	≤ 4	8
27.	Piperacillin/tazobactam	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16
28.	Piperacillin	≤ 16	≤ 16	≤ 16	64	≤ 16	64
29.	Tetracycline	8	≤ 4	8	8	≤ 4	8
30.	Ticarcillin/k-clavulanate	>64	>64	>64	>64	>64	>64
31.	Tobramycin	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
32.	Trimethoprim/ Sulfamethoxazole	>2/38	>2/38	>2/38	>2/38	>2/38	>2/38

Table 2: Minimum inhibitory concentration (MIC) of tested antimicrobials against *Pseudomonas fluorescens*. MIC values are presented in µg/mL; Gr.: Group; ESBL-a, b Scrn: Extended spectrum beta-lactamase a, b Screen; MIC values in control and treated groups were evaluated using automated Micro Scan Walk-Away® system using NBPC30 panel.

S. No.	Code	Biochemical	Type of Response					
			Gr. I	Gr. IIA	Gr.IIB, Study I	Gr. IIB, Study II		
			Control	Day 10	Day 159	Day +5	Day +10	Day +15
1.	ACE	Acetamide	-	-	-	-	-	-
2.	ADO	Adonitol	-	-	-	-	-	-
3.	ARA	Arabinose	-	-	-	-	-	-
4.	ARG	Arginine	+	-	-	-	+	+
5.	CET	Cetrimide	+	-	+	-	-	-
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-fermentation/ glucose	+	+	+	+	+	+
21.	ONPG	Galactosidase	-	-	-	-	-	-
22.	ORN	Ornithine	-	-	-	-	-	-
23.	OXI	Oxidase	+	+	+	+	+	+

24.	P4	Penicillin	+	+	+	+	+	+
25.	RAF	Raffinose	-	-	-	-	-	-
26.	RHA	Rhamnose	-	-	-	-	-	-
27.	SOR	Sorbitol	-	-	-	-	-	-
28.	SUC	Sucrose	-	-	-	-	-	-
29.	TAR	Tartrate	-	-	-	-	-	-
30.	TDA	Tryptophan deaminase	-	-	-	-	-	-
31.	TO4	Tobramycin	-	-	-	-	-	-
32.	URE	Urea	-	-	-	-	-	-
33.	VP	Voges-Proskauer	-	-	-	-	-	-

Table 3: Effect of biofield treatment on biochemical reactions of *Pseudomonas fluorescens*. -: negative; +: positive; Gr.: Group; Biochemical reactions in control and treated groups were evaluated using automated MicroScan Walk-Away® system using NBPC30 panel.

Feature	Gr. I	Gr. IIA	Gr. IIB, Study I	Gr. IIB, Study II		
	Control	Day 10	Day 159	Day +5	Day +10	Day +15
Biotype number	0204 1722	4000 0043	4000 0043/ 0004 0022	4000 0043	0204 0622	4000 2043
Organism identification	*P. fluorescens*	*Vibrio fluvialis*	*Vibrio fluvialis/ Pseudomonas* spp.	*Vibrio fluvialis*	*P. fluorescens/ putida*	*Vibrio fluvialis*

Table 4: Effect of biofield treatment on biotype number of *Pseudomonas fluorescens*. Biotype numbers and organism identification in control and treated groups were evaluated using automated MicroScan Walk-Away® system using NBPC30 panel.

Amplification and sequence determination by 16S rDNA

In order to confirm the PCR-based identification results, 16S rDNA sequence analysis was performed in biofield treated lyophilized *P. fluorescens* strain. The alignment and assessment of the gene sequences data were performed by comparing with the sequences available in GenBank database of NCBI, using the algorithm BLASTn program. The phylogenetic tree was constituted using BLAST-Webpage (NCBI). Based on nucleotides homology and phylogenetic analysis the Microbe (Sample 2A) was detected to be *Pseudomonas entomophila* (GenBank Accession Number: AY907566) with 96% identity of gene sequencing data. Ten closely related bacterial species and *P. fluorescens* were considered as Operational Taxonomic Units (OTUs) in order to investigate the phylogenetic relationship of *P. fluorescens* among other ten related species (Figure 1). Total 1482 base nucleotide of 16S rDNA gene sequences were analyzed by multiple alignments using ClustalW of MEGA3.1 program [24]. Based on the phylogenetic tree and 16S rDNA sequencing, the nearest homolog genus-species was found to be *Pseudomonas fluorescens* (Accession No. EF672049). Other closely related homologs of *P. fluorescens* can be found from the sequence alignment as shown in Table 5. Distance matrix between the 16S-rDNA sequences of 11 pathogens was analyzed based on nucleotide sequence homology using Kimura-2 Parameter. According to the data in Table 6, the lowest value of genetic distance from sample 2A was 0.003 base substitutions per site. Total 11 sequences of base substitutions per site from pairwise distance analysis were shown in Table 6.

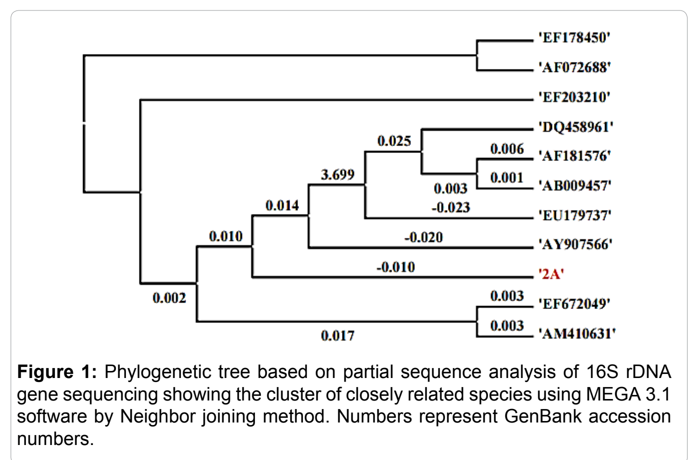

Figure 1: Phylogenetic tree based on partial sequence analysis of 16S rDNA gene sequencing showing the cluster of closely related species using MEGA 3.1 software by Neighbor joining method. Numbers represent GenBank accession numbers.

Discussion

P. fluorescens causes bacteremia and pseudo bacteremia in immunocompromised patients [25,26]. Discovering a new drug chemical moiety against resistant strain will require huge effort and time, and unfortunately, new drugs have been accompanied by the quick emergence of resistant via various natural mechanisms. However, biofield treatment on microbes has been reported as an alternate approach to improve the susceptibility pattern of antimicrobial up to a great extent.

In the preset work, authors investigated the effect of biofield treatment on lyophilized strain of *P. fluorescens* and its sustained effect was studied at day 159, followed by retreatment. The results showed alteration in antimicrobial sensitivity in case of aztreonam, cefepime, and tetracycline in different assessment days. *P. fluorescens* was highly sensitive to kanamycin, tetracycline, and ciprofloxacin at very low concentration [27]. According to Benito et al. *P. fluorescens* is susceptible to both gentamicin and Ceftazidime [28]. Gentamycin and Ceftazidime are the choice of drug used against *P. fluorescens* infection. Experimental control results were well supported with literature data [29]. According to Greenberg et al. Amikacin with aztreonam has synergistic effect against *Pseudomonas aeruginosa* and other Gram-

Alignment View	AN	Alignment results	Sequence description
	2A	0.99	Sample studied
	AF072688	1.00	*Pseudomonas mosselii* strain CIP 105259
	AF181576	0.98	*Pseudomonas monteilii*
	EF203210	0.97	*Pseudomonas putida* strain J312
	EU179737	0.98	*Pseudomonas putida* strain MG-Y2
	AY907566	0.96	*Pseudomonas entomophila* strain L48
	DQ458961	0.99	*Pseudomonas putida*
	AB009457	1.00	*Pseudomonas plecoglossicida*
	EF178450	0.98	*Pseudomonas entomophila* strain 2P25
	EF672049	0.96	*Pseudomonas fluorescens* strain Mc07
	AM410631	0.97	*Pseudomonas fluorescens* strain 9zhy

Table 5: The closest sequences of *Pseudomonas fluorescens* from sequence alignment using NCBI GenBank and ribosomal database project (RDP). AN: GenBank Accession Number. Alignment results and sequence description has been obtained from the blast results of GenBank database of National Center for Biotechnology Information (NCBI) using the algorithm BLASTn program.

Distance Matrix												
AN		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
EF203210	1	—	-2.755	-2.766	-2.760	-2.708	1	1	0.978	0.978	0.999	0.999
DQ458961	2	3.755	—	0.991	0.996	0.996	-2.755	-2.755	-2.767	-2.767	-2.703	-2.708
AF181576	3	3.766	0.009	—	0.992	0.992	-2.766	-2.766	-2.778	-2.778	-2.713	-2.719
AB009457	4	3.760	0.004	0.008	—	0.995	-2.760	-2.760	-2.772	-2.772	-2.708	-2.713
EU179737	5	3.708	0.004	0.008	0.005	—	-2.708	-2.708	-2.717	-2.717	-2.657	-2.662
EF178450	6	0.000	3.755	3.766	3.760	3.708	—	1	0.978	0.978	0.999	0.999
AF072688	7	0.000	3.755	3.766	3.760	3.708	0.000	—	0.978	0.978	0.999	0.999
EF672049	8	0.022	3.767	3.778	3.772	3.717	0.022	0.022	—	0.994	0.979	0.977
AM410631	9	0.022	3.767	3.778	3.772	3.717	0.022	0.022	0.006	—	0.979	0.977
AY907566	10	0.001	3.703	3.713	3.708	3.657	0.001	0.001	0.021	0.021	—	0.997
2A	11	0.001	3.708	3.719	3.713	3.662	0.001	0.001	0.024	0.024	0.003	—

Table 6: Distance matrix between the 16S rDNA sequences of 11 pathogens based on nucleotide sequence homology (Using Kimura-2 Parameter) of *Pseudomonas fluorescens*. AN: GenBank Accession Number. Total 1482 base nucleotide of 16S rDNA gene sequences were analyzed by multiple alignments using ClustalW program. Pairwise distance (lower left) and number of nucleotide difference (upper-right) for 16S forward and reverse primer was presented using Kimura-2 Parameters.

negative organisms [30]. Although, *P. fluorescens* is susceptible in all the groups against Amikacin before and after treatment, but found resistant against aztreonam in control (Gr. I). After biofield treatment in lyophilized as well as retreatment in revived state showed improved sensitivity pattern of aztreonam from resistant to intermediate. Also, MIC results were well supported (i.e., decreased MIC to 16 µg/mL) with improved sensitivity data as compared to control. Likewise, cefepime efficacy for the treatment of pneumonia in hospitalized patients against *Pseudomonas* infections was well reported [31,32]. The experimental results showed improve efficacy of cefepime in terms of sensitivity as well as MIC value (two-folds decrease) after biofield retreatment as compared to control. Biofield treatment on *Pseudomonas* might up-regulated the efflux membrane, or alter the function of inducible AmpC β-lactamase enzymes, which could results in altered sensitivity. Similarly, use of tetracycline against *Pseudomonas* infections has been well studied [33]. Biofield treatment showed improved sensitivity as well as decreased MIC value of tetracycline. Moxifloxacin and extended spectrum beta lactamases (ESBL) showed improved susceptibility against *P. fluorescens* with respect to control results.

Characteristic biochemical reactions of *Pseudomonas* species showed negative reaction in Voges-Proskauer, indole, and methyl red, hydrogen sulfide, glucose while positive reaction in catalase test. While some species i.e., *P. fluorescens* showed a positive oxidase test [34]. In this study, results of biochemical reactions were showed positive reaction in arginine, cetrimide, kanamycin while negative reaction in Voges-Proskauer, arabinose, colistin, rhaminose, malonate, melibiose, nitrate, galactosidase, ornithine, raffinose, sorbitol, sucrose, and tobramycin. After biofield treatment, results showed altered biochemical reactions of arginine, cetrimide, glucose, and kanamycin. Study using different clinical isolates of *Pseudomonas* spp. showed that *P. fluorescens* did not have the capacity to grow or utilize glucose in media, proof to be a real feature of pseudomonads [34]. Biofield treatment might cause some changes at enzymatic or metabolic pathway leading to significant phenotypic alteration in *P. fluorescens*, which may results in utilizing glucose in growth media. Altered biochemical reactions were results in identification of changed biotype number and species. Biotyping makes use of the pattern of metabolic activities expressed by an isolate, colonial morphology and environmental tolerances. In this experiment, biotyping was performed using automated system, results showed significant change in the biotype number with identification of new species in treated groups, and organism identified as *Vibrio fluvialis* with respect to control. Additionally, molecular methods were further studied to study and confirm the changes in species using 16S rDNA sequencing method.

Genotypic identification methods would be expected to circumvent this change in species as evidenced by altered biochemical reactions and

biotype number. Molecular assays based on 16S rDNA amplification protocol have been described. These include PCR assays and DNA amplification using standard forward and reverse 16S universal primers. 16S rDNA amplification protocol has been commonly used as a taxonomic "gold standard" in identification and determining the phylogenies of bacterial species [35]. Selective amplification using 16S rDNA was well reported to detect and differentiate *Pseudomonas* species from clinical and environmental samples [36]. Based on the BLASTn analysis, the sample 2A was identified as *Pseudomonas entomophila* with 96% similarity in gene sequence. The phylogenetic tree diagram (Figure 1) showed that nearest homolog species was found to be *Pseudomonas fluorescens.* Above results suggest that biofield treatment has significant impact on *Pseudomonas* species, which was well supported with 16S rDNA analysis.

Thus, above results indicates that biofield treatment in lyophilized as well as revived state showed significant results in terms of improved antimicrobials sensitivity, decreased MIC, altered characteristic biochemical reactions followed by change in biotype number. Study results conclude that biofield treatment has significant and sustained effect at phenotypic level for a total duration of 174 days. Study design and results showed that alterations might occur even after storage of sample in -70°C for 159 days. This suggests that Mr. Trivedi's unique biofield treatment has the ability to alter the antimicrobial sensitivity in treated pathogenic microbes even in the lyophilized storage condition for long duration. Bioelectromagnetic-based therapies and biofield therapies are energy therapies that use or manipulate the energy fields to promote health and healing. These energy therapies are well described under energy medicine by National Center for Complementary and Alternative Medicine (NIH/NCCAM). Biofield therapies are very popular in biomedical heath care systems [37]. Biofield healing treatment on pathogenic strains might involve change in cell receptor protein due to electromagnetic field. Healing therapy or therapeutic touch might modify ligand-receptor interaction, which causes alteration in phenotypic characteristics [38]. Hence, it is assumed that biofield treatment made some alteration at enzymatic or genetic level, which manifested in phenotypic alteration.

Conclusions

Improved antimicrobial sensitivity and decreased MIC value of aztreonam, cefepime, moxifloxacin, and tetracycline in biofield treated *Pseudomonas fluorescens* showed significant impact of Mr. Trivedi's biofield energy treatment. This approach can be used as a treatment approach in complementary and alternate medicine. Significant alteration in antimicrobial data was well supported with altered biochemical reactions along with biotype number. Molecular approach using standard 16S rDNA analysis showed that biofield treatment has significant impact on *P. fluorescens*, and sample identified as *Pseudomonas entomophila* with 96% identity using gene sequencing data. However, the closest homolog species was detected to be *Pseudomonas fluorescens.* Based on these results, it seems that Mr. Trivedi's biofield energy treatment could be used as better alternate of existing drug therapy in future.

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