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Applicability of Genotypic and Phenotypic Methods to the Assessment of the SKEO Effect on MexXY-OprM and MexEF-OprN Efflux Pumps in MDR and Clinical Isolates of *Pseudomonas aeruginosa*

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In this study to be tried that check out the effect of *Satureja khuzistanica* essential oil (SKEO) against *mexXY-oprM* and *mexEF-oprN* efflux pumps genes expression in MDR and clinical isolates of *Pseudomonas aeruginosa*. For notice of the genes expression amount before and after of confronting with SKEO, genotyping and phenotyping methods were used. In phenotyping method was applied MIC technique with specific antibiotic markers (Norfloxacin and Imipenem for MexEF-OprN and Gentamicin for MexXY-OprM). In genotyping method was used of RT-PCR technique. The amount of SKEO·s MIC compared to antibiotics were much less. The MIC of antibiotic markers and intensity of bands on gel electrophoresis after confronting decreased. Reduce in MIC of antibiotic markers and as well as in the bands intensity show that SKEO decreased expression pumps and both methods confirm each other. Therefore, SKEO could be an important factor in the reduction of antibiotic resistance in the *P. aeruginosa* isolates. According to these results, we expect be applied SKEO as a supplement therapy in future infections.

Key words: Genotypic and phenotypic methods, SKEO Effect, *Pseudomonas aeuroginosa*.

P. aeruginosa is a major cause of nosocomial infections and is famous because of its multiple resistances to antibiotics[1]. MDR *P. aeruginosa*, expand resistance by a combination of low permeability property and efflux pumps[2, 3]. Several pumps are present in the *P. aeruginosa* structure. The RND family is one of the most important efflux pumps families[4] which obtain the energy from the proton motive force (5). The *P. aeruginosa* chromosome encompasses several genes for the RND efflux pumps, of which MexXY-OprM, MexEF-OprN, MexCD-OprJ and MexAB-OprM are significant clinically [5, 6].

The increased prevalence of antibiotic resistance in *P. aeruginosa*[7], expensive novel antibiotics for overcoming emerge problems and adverse side effects related to antibiotics are the reasons that the use of alternative drugs as a replacement for synthetic antibiotics is increasing[8]. Many herbs such as *Satureja khuzistanica (SK)* develop antimicrobial property because of their essential oil components[9]. *Satureja khuzistanica* is a native plant in the southern regions of Iran.

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Chemical analysis proved that the original composition of *SK* is Carvacrol (93%)[10]. Fundamental mechanism *Satureja khuzistanica* essential oil (SKEO) in damaging to bacteria is because of Carvacrol and Thymol. These materials that are characterized by high hydrophobicity, able to separate the broad spectrum of bacterial cell wall lipids and in this way increase the permeability of the membrane. Bacterial cell membrane dysfunction resulted in the removal of ions, nucleic acid and adenosine triphosphate (ATP)[11]. Proton pump was inactive and accumulates antibiotics and damaged to bacteria[12]. Following the SKEO direct damage to bacteria through efflux pumps destruction, it may also be as a stressful and indirectly inhibit the expression.

Right now, to evaluate the efflux pump activity used phenotypic and genotypic approaches. Because according to previously noted by others, phenotypic-based methods generally deliver vague outcomes, probably due to the co-expression of resistance mechanisms[13]. On the other hand, genotypic methods do not certainly supply data on the ultimate expression of the gene (14). In addition, selection of primers and probes in the genotypic methods can introduce severe bias. Therefore, each of these methods alone, not confirms correct expression rate of pumps. In this study, we attend to this issue by combining phenotypic and genotypic approaches.

The object of this research is to assess antimicrobial activity of SKEO against two important Mex efflux pumps include MexXY-OprM and MexXY-OprM in MDR isolates of *P. aeruginosa* with genotypic and phenotypic methods[9]. For this purpose, in phenotypic method (MIC) to assess the extent of damage to the pumps used of specific markers for each pump and in the genotypic method for the study of inhibiting the expression of efflux pumps used of reverse transcription PCR (RT-PCR) method.

MATERIALS AND METHODS

Bacterial isolates and growth conditions

The nosocomial isolates were randomly isolated from different burned patients during a period of 2 months between July and August 2014 that were hospitalized in different departments of the Shahid Motahari Hospital in Tehran. Subsequently, samples were grown on blood agar, MacConkey agar and Pseudomonas agar (Oxoid, England). After incubation, Gram staining and oxidase test were done on pure cultures. Just Gram-negative bacilli and oxidase-positive isolates were chosen for standard biochemical tests.

Antibacterial susceptibility testing

This study is based on the CLSI 2014 standards and instructions and CLSI criteria were applied in the interpretation of the results. All media, disks and antibiotic powders were adjusted by standard strain (*Escherichia coli* ATCC25922) till ensuinge that the tests executed appropriately.

Inhibitory effect of antibiotics by disc diffusion method

Disks were prepared from Padtan Teb Co., Tehran, Iran. Susceptibility determination was done by the disc diffusion technique of Kirby Bauer with multiple anti-microbial and anti-Pseudomonal classes disks[15] but isolates were entered into the study that both were non-duplicate (isolates with different resistance patterns) and were resistant, at least to three antibiotics, Imipenem (Imi, 10 µg/disk), Gentamicin (Gen, 10 µg/disk), and Norfloxacin (Nor, 10 µg/disk). Finally, two clinics isolate of MDR *P. aeruginosa* were applied in this study that is cited in Table 2.

Therefore, one colony of each isolate was suspended in MHB and was grown overnight at 37°C, then the bacterial suspensions were modified by comparing with to 0.5 McFarland standard and authenticated by measuring the absorption using a spectrophotometer at 625nm. Then cell suspensions inoculated to Petri dishes with 20 ml of Mueller- Hinton Agar (MHA). Application of disks to inoculated plate agar was done. The inoculated dishes were incubated at 37°C under aerobic conditions. At the end of 24h incubation, the inhibitory effect was measured by calculating the diameter of non-growth diffusion zones in millimeters and the average were indicated as the results of three determinations.

Determination of MIC's SKEO with macrodilution broth method

After the MDR isolates were recognized by disk diffusion method, the efficiency of SKEO was determined against clinical isolates by the broth macrodilution method. At first, for make various dilutions of essence, a stock of SKEO was prepared with ratio 10% in it's particular solvent,

Dimethyl sulfoxide (DMSO), The stock oil was kept away from heat and direct light. Then, were added to ten tubes 40-130 µl of the stock respectively and was promoted volume of each tube to the 1 ml with Mueller- Hinton broth (MHB).

On the other hand, for gain inoculum suspension, one colony of every bacterial isolate was selected with a loop, then inoculated in 5 ml MHB and incubated at 37°C for 18–24 h under aerobic conditions with continuous shaking at 100 rpm[7]. After the incubation, the inoculum suspension was diluted with MHB until achieving 1×10^6 cfu/ml bacterial suspensions for all tubes. Then 1 ml of different concentrations of essence was added to tubes comprising 1 ml of bacterial suspensions. The final volume and concentration in each tube was 2 ml and 5×10^5 cfu/ml respectively. In each series, positive control was inoculums but no SKEO and negative control was SKEO but without inoculums. The contents of the tubes were mixed and incubated at 37°C for 24 h under aerobic conditions. Since SKEO makes the hazy MHB and lowest concentration that prevented the visible growth is not readable, therefore after incubation, 20 µl from each test tube was subcultured onto MHA plates. After 24h incubation, dilution before the negative subculture dilution or dilution before the subculture dilution that only giving one colony were defined as MIC. The tests were carried out in three replicates but in three various days. Then cultured a dilution lesser than MIC's SKEO on MHA for any isolates. These obtained colonies of confronting preserved for next stage.

Determination of MIC of resistance marker with macrodilution broth method

Three antibiotics were applied as phenotypic markers and entered into this study: Nor and Imi for MexEF-OprN and Gen for MexXY-OprM (13). MICs of these antibiotics were determined against the obtained colonies of the before and past of confronting with SKEO.

The inoculums were prepared by culturing in MHB and 3-4 h incubation for each isolate, moderated to the turbidity of 0.5 McFarland Standard. This suspension is attenuated in MHB to be obtained a final concentration of 10^6cfu/ml. For each antibiotic were ready to fold serial dilutions, between 0.5 to 512 µg/ml in MHB. By adding 1 ml of each dilution to 1 ml of bacterial suspension, the total volume of reaction reached to 2 ml. In case resistance to 512 µg/ml concentration, the serial dilution was extended to 8192 µg/ml concentration. All antibiotic powders were prepared from Sigma- Aldrich. MIC was measured with eyes.

RNA Extraction and cDNA Synthesis

For analyse the expression of efflux pump genes, the isolates before and past of confronting with SKEO, were grown in Luria -Bertani (LB) broth and then were harvested at 18-24h of growth (1×10^9 bacterial cells) by centrifuge. The cells were used directly for RNA extraction applying total RNA extraction kit, in an RNase-free environment (16) according to the manufacturer's protocol (Sinaclon Bioscience, Karaj, Iran). All RNA samples subsequently were treated with 1U of RNase-free DNaseI (Sinaclon Bioscience, Karaj, Iran) per 1µg of RNA at 37°C for 0.5 h to inhibit contamination with DNA. The quality of extracting RNA was determined by electrophoresis of 5 µl the RNA on a gel agarose 1% (Sinaclon Bioscience, Karaj, Iran), visualized with a transilluminator. In addition, the lack of genomic DNA remaining was confirmed by PCR(5).

Specific primers were designed by Genscript software available at https:// www.genscript.com/ssl-bin/app/primer. The applied primers to amplify and identify *mexY* and *mexE* genes were displayed in table 1. The *gyrA* (housekeeping gene) was used as a control[16].

The differences in the expression proportion of *mexY*, *mexE* and *gyrA* genes were recognized by the RT-PCR method. For this purpose, For all RNA extracted, cDNA was synthesized by using 2-step RT-PCR kit (Vivantis, Malaysia) (16). Each 10 µl RNA-primer mixture contained 2 µg of total RNA, 10 pmol of each reverse and forward primers and 1 µl of dNTP mix (10 mM each). The RNA-primer mixture was incubated at 65°C for 5 min and rapidly chilled on ice for 2 min. In another microtube, cDNA synthesis mix was prepared according to following orders. For this purpose, 1 µl of M-MuLV Reverse Transcriptase, 2 µl of 10x Buffer M-MuLV and 7 µl of nuclease free water were mixed. Eventually, in another microtube, were mixed 10 µl of the RNA-primer mixture and 10 µl of the cDNA synthesis mix and then combinational microtube was incubated at 42°C for 60 min and, therefore, cDNA was synthesized. The synthesized cDNA were both

immediately used in RT-PCR assay and stocked at -20°C.

RT-PCR and DNA Sequencing

cDNA from each bacterial isolates was separately applied as DNA template in RT-PCR. The RT - PCR reaction was performed in a 25 µl volume containing 2 µl cDNA (10 pg – 1 µg/µl), 10 pmol of each primer, 12.5 µl of ready master mix (Sinaclon, Karaj, Iran) and sterile deionized water added to 25 µl. The PCR circumstances were as follows: pre-denaturation for 3 min at 94°C , 30 cycles of denaturation for 30s at 94°C, annealing for 30s at 57°C and extension for 30s at 72°C, continued by a final extension for 5 min at 72°C[16].

Amplified products (5 µl) were separated by 2% agarose gel electrophoresis in TBE buffer. A 100 base pair ladder (Fermentas, Germany) was applied. After electrophoresis gel stained with ethidium bromide and imagine. The one PCR amplicon of each gene was purified from the gel using GF-1 Gel DNA Recovery Kit (Vivantis, Malaysia) and with the PCR primers sent for sequencing at Pishgam Biotech Company, Tehran, Iran. The DNA sequences received were evaluated with the related sequences in the Pseudomonas Genome Project[17].

RESULTS

Determination of MICS's SKEO and antibiotic markers against clinical isolate

To attain accurately the antimicrobial properties of SKEO for possible application in patient treatment, determination of MICS's SKEO was necessarily performed on both clinical and MDR isolates[7]. The MICs of SKEO against two clinical isolates were summarized in Table 2. The results proved similar effects of SKEO on the tested bacterial isolates (MIC= 8 µl/ml) (Tables 2) and both the tested bacteria showed sensitivity to SKEO.

As well as, the MIC values of antibiotic markers against the clinical isolates before and after confronting with SKEO's were shown in Table 2. The MIC results of examining antibiotics were between about 4 – 4096 µg/ml. The lowest MIC reduction was seen for Imi in PA10 with 3 fold reduction.

Although SKEO in the initial contact both isolates had a similar effect (MIC=8 µg/ml) but was caused a 1-3 fold reduction of the MICs of Imi, Nor and Gen that this topic can be a sign of low expression of MexEF-OprN and MexXY-OprM[13].

Table 1. The list of primers and their sequences that were used to amplify the study genes by using RT-PCR

Source	Size	Sequence	Primer
This study	108 bp	GCCCAACGACATCTACTTCA	*mexY* (F)
		ATGCCTTCCTGGTAATGGTC	*mexY* (R)
This study	131 bp	TCCTCAAGTACGTCGAGCTG	*mexE* (F)
		GACCTGGTTGTCGAGGAAGT	*mexE* (R)
This study	121 bp	GGTCTGGGCATAGAGGTTGT	*gyrA* (F)
		GAAGATCGAGGGTATTTCCG	*gyrA* (R)

Table 2. Antimicrobial susceptibility for resistance markers and SKEO and intensity of expression the MEX efflux done by RT-PCR in the MDR clinical isolates of *P. aeruginosa*

Clinical isolates	Intensity of expression		MIC (µg /ml) Imi (For *mexE*)		Nor (For *mexE*)		Gen (For *mexY*)		SKEO
	-SKEO	+ SKEO	-SKEO	+ SKEO	- SKEO	+ SKEO	- SKEO	+ SKEO	
PA9	Stronger	Weaker	16 (R)	4 (S)	32 (R)	8 (I)	4096 (R)	2048 (R)	8
PA10	Stronger	Weaker	32 (R)	4 (S)	16 (R)	4 (S)	2048 (R)	1024 (R)	8

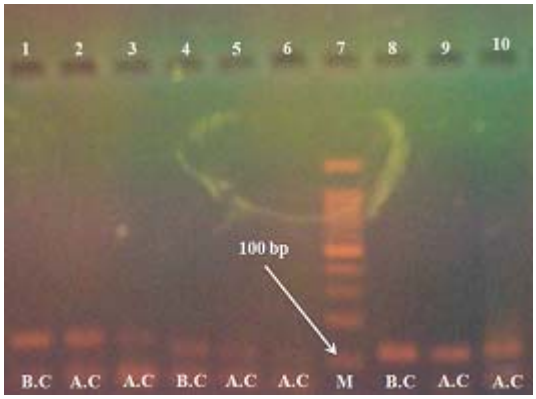

Fig. 1. PCR amplicons of 2 important Mex pumps and internal control generated by RT-PCR. Lane 1 - 3 bands for *mexE* (131 bp), Lane 4 - 6 bands for *mexY* (108 bp), Lane 7 M, molecular weight marker; Lane 8 - 10 bands for *gyrA* (121 bp). Lane 1, 4, 7 bands for before confronting (B.C). Lane 2, 3, 5, 6, 9, 10 bands for after confronting (A.C)

Nevertheless, SKEO couldn't reduce MIC to sensitivity bound only for one marker (Gen).

Expression determination of the Mex efflux pumps by RT-PCR

When the cDNA from both the clinical isolates was used for the template, all genes were expressed pre and post of confronting with SKEO and their specificity was confirmed by DNA sequencing analyses. The severity of expression of the Mex efflux pumps is shown in Table 2 and figure 1. The results of RT-PCR determined that the expressions of *mexE* and *mexY* genes remarkably decreased after of confronting (Figure 1). As forecasted, expression of *gyr*A as an internal control gene was constant throughout the study.

DISCUSSION

Just a few reports have been noted on the antibacterial specify of SKEO against *P. aeruginosa*. In comparison to previous studies[8, 9, 18], our conclusions shown a difference of antibacterial activity of SKEO against *P. aeruginosa*. It might be described by the different contents and composition of active constituents in SKEO (7, 19). Furthermore, the methods used to measure the antibacterial activity, selection of bacterial strains and the amount of their sensitivity, mass of inoculums, time and temperature incubation may have influence in the experimental results[19, 20].

Comparing the effect of SKEO with markers against the tested bacterial isolates, the activity of SKEO is about 2-512 fold more than of the antibiotics. On the other hand, the SKEO revealed powerful antibacterial activities in inhibiting the growth of all tested compared to the resistance markers.

In all the clinical strains, a good relationship was observed between phenotypic and genotypic results (Table 2 and figure 1). As in RT-PCR, after confronting, genes expression and MIC of the respective antibiotics are lower than before confront. But although the in RT-PCR, expression level *mex*Y is weaker than *mex*E, on the contrary, the MIC of the relevant antibiotic marker (Gen) is much higher than the MIC of Nor and Imi markers. Then this issue has proved that other factors besides efflux pump, involved in resistance. Therefore, cannot simply be used phenotype test to predict the actual expressed-Mex efflux pumps.

In phenotypic recognition, the antibiotics used are unique substrates for each of Mex pumps[21]. MexXY-OprM and MexEF-OprN participate in innate and acquired resistance respectively[22-24]. As based on previously suggested, for MexEF-OprN, Imi and Nor were involved as indirect signs of the pump[13]. Simultaneous expression of down-regulated OprD and up- regulated MexEF-OprN will cause resistance to carbapenems in the up-regulated MexEF-OprN mutant strain[25]. In this study, the addition of SKEO caused 2-3 fold-reductions of Imi and Nor MIC in both isolates. Nevertheless, the OprD expression and its real involvement of Mex-EF-OprN in the up-regulated MexEF-OprN isolates weren't examined. Other than Imi, the marginal effect (*i.e.* 2 fold reductions) on antibiotic susceptibility was observed in Nor for both isolates. The addition of SKEO and 1-3 fold-reductions of MIC's antibiotics demonstrated that the share of the Mex systems in resistance level is diverse and noted the existence of other resistance mechanisms.

There are several specific methods to directly prove the proteins and genes encoding the for efflux pumps. Quantitative RT-PCR technology (RT-qPCR) is a rapid method with excellent sensitivity and specificity, low contamination risk, simultaneous run of several reactions and high throughput [26]. However, many

probes are needed for concomitant detection of many Mex genes, leading to increased cost[27]. Study of the Mex pump based on the western blot technique has depended on applying antibody. This method is complicated, time - consuming and the particular antibodies to the Mex proteins are not easily accessible[5]. In contrast, RT-PCR is less expensive but laborious. Importantly, RT-PCR allows us to quickly observe the amplification products and easily is feasible in the clinical laboratory where a sophisticated real- time PCR device is not available[27]. Because the Mex expression quantity isn't measured, the effectiveness of RT-PCR may be less in comparison with real-time RT-qPCR. But, several studies revealed no correlation between the quantity of expression and resistance in *P. aeruginosa* isolates[27, 28]. Therefore, the determination of transcription quantity is not at all times necessary for usual diagnosis[27].

In summary, this data may be helpful for rationalizing the choice antibiotic dosing, devising antimicrobial formulations and clinical utility of SKEO as inhibitors of efflux pumps for protecting patients against infection with MDR of *P. aeruginosa* isolates. Thus, the data acquired in this study can be interestingly used as a good complementary and confirmatory data for the formerly published studies. Of course for the correct recognition of resistance mechanisms in these organisms, the larger number of strains and effluxes must include in the study. As well as, additional clinical and trial research is essential to entirely confirm the mentioned results for medical purposes.

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