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The retrieval of antibiotic sensitivity by 2-phenoxyethanol adapted-multi drug resistant (MDR) clinical isolate of *Pseudomonas aeruginosa*

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2-Phenoxyethanol (2-PE) is a biocide used as a preservative in pharmaceutical, cosmetic and perfumery formulations. It is known for its rapid bactericidal effect against a wide range of bacteria. In the present study, clinical samples of multi drug resistant (MDR) *Pseudomonas aeruginosa* and *Escherichia coli* were grown in an increasing sub-minimal inhibitory concentration of 2-PE to observe if adaptation could be obtained and consequently retrieval of antibiotic susceptibility to clinically used antibiotics. Minimal inhibitory concentration (MIC) of 2-PE was determined for both MDR *E. coli* and *P. aeruginosa*. Adaptation to the biocide was attempted by repeated sub- culturing of the isolates in increasing sub-minimal-inhibitory concentration of the biocide. *E. coli* was able to grow in the presence of sub-MIC of 2-PE for two passages only and a slight rise in MIC was observed. MDR *P. aeruginosa* rapidly obtained an adaptive resistance towards the biocide with an increase in MIC from 5.4 to 13.7g%. Antibiotic sensitivities for both, MDR and MDR-2-PE adapted *E. coli* and *P. aeruginosa* were detected. MDR2-PE resistant *P. aeruginosa* showed significant improvement in antibiotic sensitivity while no change was observed with *E. coli*. Our results suggest an intriguing species specific link between biocide resistance and changes in antibiotic susceptibilities, findings that could be utilized in controlling the spread of antibiotic resistance and ambitiously improving the activity of some existing antibiotics.

Key words: *Pseudomonas aeruginosa,* 2-phenoxyethanol, antibiotic resistance.

INTRODUCTION

The role of microbicides in emerging bacterial resistance towards antibiotics is still controversial. Several studies have pointed out that the increase in the use of biocides in domestic settings as well as hospital environments furnishes for the selection of antibiotic resistant strains (Aiello, 2005; Braoudaki and Hilton, 2004; Loughlin et al., 2002; Walsh, 2003).On the other hand, adaptive resistance to some biocides has been reported by some researchers to reduce antibiotic resistance (Abdel Malek 2009; Joynson et al., 2002). Benzalkonium chloride (BC) adaptation for example, has been described not only for laboratory strains, but also for clinical isolates of

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Abbreviations: BC, Benzalkonium chloride; **MIC,** minimum inhibitory concentration; **2-PE,** 2-phenoxyethanol; **MDR,** multi drug resistant; **DMF,** dimethylformamide; **NB,** nutrient broth; **MHB,** Mueller Hinton Broth.

Pseudomonas aeruginosa. Clinical isolates from cystic fibrosis patients were sub-cultured into increasing concentrations of benzalkonium chloride and adaptation to BC was accompanied by a decrease in minimum inhibitory concentration (MIC) to several antibiotics and accelerated the loss of resistance to imipenem (Joynson et al., 2002). BC was even suggested to be used in hospital hygiene to help overcome antibiotic resistance in clinical environment (Joynson et al., 2002). It is generally accepted that in contrast to chemotherapeutic agents, biocides have multiple target sites within the microbial cell and the overall damage to these target sites results in the bactericidal effect. However, the use of biocides at sub-lethal concentrations have been found to result in adaptive resistance to these biocides and cross resistance to antibiotics (Braoudaki and Hilton, 2004; Fraise, 2002; Hegstad et al., 2010; Karatzas et al., 2007). Since biocides have multiple mechanisms of action within the cell, adaptations to variable biocides by different microbes will result in variable alterations in antibiotic susceptibility, an issue that mandates further investigation into its aspects (Aiello et al., 2007).

E. coli O157 strains for example acquired high levels of resistance to triclosan after only two sub lethal exposures and when adapted they repeatedly demonstrated decreased susceptibilities to various antimicrobial agents, including chloramphenicol, erythromycin, imipenem, tetracycline, and trimethoprim, as well as to a number of biocides (Braoudaki and Hilton, 2004). Whereas *P. aeruginosa* had shown altered antibiotic susceptibility after adaptation to various biocides (Abdel Malek and Badran 2010; Walsh et al., 2003). Mechanisms of biocide resistance were summarized by Maillard (2010), in a recent review to be altering between changes in the outer membrane permeability, expression and/or over expression of multidrug efflux systems or alteration in the target site (Maillard, 2010). Antimicrobials can also act as triggers of the microbial stress response seen in drug resistance (Poole, 2012). Thus, by the same mechanism, stress response towards biocides could add to some microbes ability to resist antibiotics.

2-phenoxyethanol (2-PE), a powerful antimicrobial that is used as a preservative in pharmaceutical, cosmetic and perfumery formulations, has a rapid bactericidal effect against a wide range of bacteria (including *P. aeruginosa*) and has been used as a general antiseptic, bladder irrigant and in the treatment of wounds and burns (octenidine dihydrochloride) (Hubner et al., 2010; Wilson et al., 1990). 2-PE affects a multitude of intracellular targets (Beveridge et al., 1991; Denyer, 1995). At sub lethal biocide levels, a variety of concentration-dependent inhibitory processes take place, from actions such as potassium–proton antiporters and respiration uncouplers to competitive inhibition of NADH binding by malate dehydrogenase and slowed DNA biosynthesis relative to general anabolism(Russell, 2003).

Previous work by Abdelmalek and Badran (2010) had shown that 2-PE adaptation of *P. aeruginosa* PAO1 involved outer membrane alterations but did not involve resistance nodulation cell division (RND) efflux pumps (Abdelmalek and Badran, 2010), and was accompanied by a reduced resistance to some antibiotics. PAO1 *P. aeruginosa* is a laboratory strain that has been studied extensively in the field of biocide resistance (Kern et al., 1994; Maillard, 2010; Winder et al., 2000). It has been found to adapt to most biocides and this adaptation resulted from outer membrane protein alterations, fatty acid composition changes or efflux mechanisms (Abdel Malek et al., 2009; Abdelmalek and Badran, 2010; Jones et al., 1989; Strateva and Yordanov 2009). In this study, clinical isolates of MDR *P. aeruginosa* and *Escherichia coli* collected from immune compromised patients are subjected to sub inhibitory concentrations of 2-PE to investigate the possibility of adaptation to this biocide and whether antibiotic sensitivity will be affected.

MATERIALS AND METHODS

Bacterial cultures

Two multi drug resistant (MDR) isolates *E. coli* and *P. aeruginosa* were obtained from KHCC (King Hussein Cancer Center, Amman, Jordan) and were sub-cultured on nutrient agar and maintained by subculturing in Nutrient agar slopes. These cultures were used as the 2-PE-sensitive strains. Passaged cells were preserved at -70°C in glycerol.

Controls

The following strains were used as controls*: E. coli* ATCC 25922 wild type, a kind gift from Specialty Hospital, Amman, Jordan. *P. aeruginosa* PAO1. (NCIMB 10548), obtained from National Collection of Industrial, Marine and Food bacteria (NCIMB) (Aberdeen,UK).

Chemicals

2-PE (Sigma-Aldrich) was dissolved in dimethylformamide (DMF), and variable concentrations were prepared in sterile distilled H_2O and kept in amber glass bottles at 4°C until used within a week from preparation. Antibiotic discs of: Gentamicin (CN) 10 μg, amoxacillin (AML) 25 μg, doxycyclin (DO) 30 μg, amikacin (AK) 30 μg, rifampicin (RD) 5 μg, norfloxacin (NOR) 10 μg, vancomycin (VA) 30 μg, cefotaxime (CTX) 30 μg, cephalexin (CL) 30 μg , ofloxacin (OFX) 5 μg, chloramphenicol (C) 30 μg, tobramycin (TOB) 10 μg, azithromycin (AZM) 15 μg, sulphamethoxazole (SXT) 25 μg, ticar/clav (TIM) 85 μg, ciprofloxacin (CIP) 5 μg, cefixim (CFM) 5 μg, minocyclin (MH) 30 μg, aztreonam (ATM) 30 μg, ceftizoxime (ZOX) 30 μg, piperacillin (PRL) 100 μg, ceftazidime (CAZ) 30 μg, ampicillin (AMP) 10 μg, imipenem (IPM) 10 μg, nitrofuranton (F) 300 μg, were purchased from Oxoid (Germany).

Methods

Determination of MIC of 2-PE was done using the tube dilution method (Bloomfield, 1991). MICs were determined in Mueller Hinton Broth (MHB) medium (9.0 ml) with an inoculum of 100 µL from an overnight culture in the same medium grown at 37°C in a

Table 1. Minimal Inhibitory Concentration (MIC) g% of 2-PE for *E. coli* and *P. aeruginosa* strains.

Microbial strain	MIC g%
E. coli 25922	4.4
MDR *E. coli* P0*	4.4
MDR *E. coli* P1	5.3
MDR *E. coli* P2	5.3
P. aeruginosa PAO1	3.3
MDR *P. aeruginosa* P0	6.1
MDR *P. aeruginosa* P1	6.8
MDR *P. aeruginosa* P2	8.8
MDR *P. aeruginosa* P3	11.3
MDR *P. aeruginosa* P4	13.7

*PN, Number of subcultures (passages) in a sub-minimal inhibitory concentration of 2-PE; 2-PE, 2- phenoxyethanol; MDR, multi drug resistant.

shaking incubator (200 oscillations/ min). A volume (less than 900 µL) of biocide at known concentration was applied to each dilution tube. The final volume of each tube was adjusted to 10.0 ml with the addition of sterile distilled water. The tubes were incubated for 48 h at 37°C and observed for growth at 19 and 48 h. MICs were estimated as the lowest concentration of biocide that inhibited growth.

Induction of biocide adaptation

2-PE adaptation experiments were performed according to Abdelmalek and Badran (2010). Briefly, fresh *E. coli* and *P. aeruginosa* cultures were prepared by inoculating 25 mL of nutrient broth (NB) with 250 µL of an overnight culture of the isolates (adjusted to 0.7 optical density(O.D) of A470), and adding an aliquot of 2-PE that is equal to ¼ of the previously determined MIC. This culture was considered passage 1 (P1). Once the MIC value of this passage was re-determined and density adjusted, 250 µL of this culture was inoculated in fresh 25 mL of N.B media containing ¼ of the newly determined MIC. This procedure was repeated for several successive passages. The experiments were performed in triplicates, results being the mean of the three MICs determined.

Cross-resistance to antibiotics

Wild type *E. coli* ATCC 25922, MDR and 2-PE-adapted MDR E. *coli* (P2) cultures as well as MDR *P. aeruginosa* and 2-PE- adapted MDR *P. aeruginosa* (P4) were freshly prepared and inoculums size was adjusted to O.D of 0.7 at A470. Antibiotic sensitivity was determined using the disc diffusion method: bacterial cultures were seeded on Mueller Hinton Agar and antibiotic discs were applied; the inhibition zones were measured in millimeters.

Statistical analysis

Non-parametric Mann-Whitney U test was used to compare MIC values and antibiotic sensitivities of wild type and MDR strains.

RESULTS

Minimal inhibitory concentrations and adaptation experiments

Minimal inhibitory concentration of 2-PE was determined for wild type and MDR strains of both *E. coli* and *P. aeruginosa.* MIC of the wild type *E. coli* ATCC was 4.4 g% and of the MDR strain was 4.4 g%. MDR *E. coli* was able to grow for two passages only in the presence of sub MIC of 2-PE. A slight increase in MIC of passage 1 was detected. The MIC of 2-PE obtained was 5.3 g% that was stable in passage 2 (Table1). On the other hand, the 2-PE MIC of MDR *P. aeuginosa* was significantly ($p < 0.05$) higher (6.1g %) than 2-PE MIC of the control strain (PAO1) (3.3g %). MDR *P. aeruginosa* adapted rapidly to the biocide with an almost two fold increase in MIC by passage 4 (P4). The MIC increased significantly ($p < 0.05$) from 6.1 g% for the MDR strain to 13.7 g% by passage 4 (Table 1). Adaptation experiments could have been pursued for higher values in MIC but since this was not the purpose of this research the authors considered a two fold increase in MIC to be adequate.

Antibiotic sensitivity testing

The susceptibility of MDR and 2-PE adapted MDR strains of *E. coli* and *P. aeruginosa* for clinically used antibiotics was tested using the disc diffusion method. Results are presented in Tables 2 and 3, respectively. *E. coli* strain MDR and 2-PE exposed MDR did not show any significant change in antibiotic susceptibility (Table 2), except for both ciprofloxacin and doxycyclin to which the 2-PE exposed MDR strain expressed significant increased sensitivity. However, the situation was different with *P. aeruginosa.* The antibiogram of the MDR strain (Table 3) compared to the antibiogram of MDR 2-PE adapted strain displayed significant change ($p < 0.05$) from resistance to sensitivity for 15 out of 25 tested antibiotics and significant increase ($p < 0.05$) in susceptibility for 6 out of 25. On the other hand, there was no significant change in susceptibility for both tobramycin and azithromycin, from 13.6 to 20 mm and from 25 to 18 mm, respectively. Aztreonam stood out with a significant ($p < 0.05$) decrease in the zone of inhibition from 18.6 to 0 mm, being the only case of resistance.

DISCUSSION

Several reports have indicated that adaptation to some biocides could lead to antibiotic resistance (Braoudaki and Hilton, 2004 ; Carson et al., 2008; Fraise, 2002; Hegstad et al., 2010; Joynson et al., 2002). In a previous work (Abdel Malek and Badran, 2010) adaptation of PAO1 strain of *P. aeruginosa* to 2-PE resulted in increased susceptibility to some antibiotics. A question presented itself in light of these findings; what if clinical MDR isolates were exposed and adapted to 2-PE, would this improve antibiotic susceptibility? This question was investigated in the present study. Results have shown that adaptation to 2-PE was species specific; MDR *E. coli* was not able to adapt to sub-minimal inhibitory concentrations of 2-PE. A slight increase in 2-PE MIC was observed

Table 2. Comparison between antibiotic susceptibilities (as measured by zone of inhibition in (mm)of MDR clinical isolate of *E.coli* and the same isolate after exposure to 2-PE.

Antibiotic	Zone of inhibition (mm) (±SD)		
	MDR *E. coli*	2-PE exposed MDR *E. coli*	*P-value
Gentamicin	21.6(.05)	21(.1)	
Amoxacillin	0(0)	2.6(.46)	
Doxycyclin	15.6(.05)	20.6(.46)	<0.05
Amikacin	21(.17)	20(0)	
Rifambicin	0(0)	0(0)	
Norfloxacin	0(0)	0(0)	
Vancomycin	0(0)	0(0)	
Cefotaxime	0(0)	0(0)	
Cephalexin	0(0)	0(0)	
Ofloxacin	0(0)	0(0)	
Chloramphenicol	21.3(.15)	22.3(.25)	
Tobramycin	20.3(.05)	20(0)	
Azithromycin	13.6(.05)	13.3(.05)	
Sulphamethoxazole	24.6(.05)	25 (.17)	
Ticar/clav	7.6(.05)	0(0)	
Ciprofloxacin	0(0)	10.3(.11)	<0.05
Cefixim	0(0)	0(0)	
Minocyclin	17(.1)	21.3(.58)	
Aztreonam	15(.1)	13.6(.05)	
Ceftizoxime	0(0)	0(0)	
Piperacillin	11.6(.15)	9.6(.05)	
Ceftazidime	0(0)	0(0)	
Ampicillin	0(0)	0(0)	
Imipenem	25.3(.05)	23(.17)	
Nitrofuranton	20.3(.15)	20(0)	

*P-value calculated using a non-parametric Mann-Whitney U test.

(Table 1) beyond which the bacteria were not able to adapt or grow. On the other hand, MDR *P. aeruginosa* adapted rapidly to 2-PE with a significant two fold increase in MIC (from 6.1 to 13.7 g%). Such finding is compatible with what has been documented earlier by Bailey et al. (2009) and Braoudaki and Hilton (2004).

When the 2- PE exposed MDR and the MDR *E. coli* strains were tested for their sensitivity to antibiotics, susceptibility patterns did not vary between the two strains (Table 2). Statistically speaking, we observed significant ($p<0.05$) increase in susceptibility to ciprofloxa-cin and doxycyclin as seen by increase in the zone of inhibition from 0 to 10 mm and from 15.6 to 20.6 mm, respectively. However the change in sensitivity is very little and does not mean that 2-PE exposed MDR- *E.coli* became susceptible, since the minimum zone of inhibition required for these antibiotics are 21 mm for ciprofloxacin and 16 mm to doxycycline (Wikler, 2008). Resistance towards hydrophilic fluoroquinolones, (ciprofloxacin, ofloxacin and norfloxacin) in *E. coli* is controlled by the norA gene (Yoshida et al., 1990). Thus, if the suggested efflux pump mediated by the norA gene was inhibited by exposure to 2-PE, this should result in increased sensitivity towards these three floroquinolons. But the effect was only observed towards ciprofloxacin and not the other two floroquinolons. On the other hand, doxycyclin and other antibiotics are substrates to the same efflux pump in MDR *E. coli* isolates (Kern et al., 1994), nevertheless, no change in other antibiotics' sensitivities was observed (Table 2). These results necessitate more investigation.

In comparison, 2- PE adapted MDR *P. aeruginosa* demonstrated a remarkable change in antibiotic susceptibility. Adaptation to 2-PE was accompanied by a significant ($p<0.005$) complete reversal in antibiotic sensitivity profile from resistance to sensitivity towards 14 out of 25 antibiotics tested. These antibiotics were penicillins (amoxacillin, ampicillin, ticar/clav) cephalosporines (cefotaxime, cephalexin rifampicin, cefixim), quinolones (norfloxacin, ciprofloxacin, ofloxacin,), vancomycin, sulphamethoxazole, nitrofuranton and imipenem. Moreover, 6 out of 25 antibiotics, showed significant ($p<0.005$) increase in sensitivity and these were tetracycline (doxycyclin, minocycline), gentamycin, piperacillin, ceftazidime and chloramphenicol. On the other hand, sensitivity to aztreonam

Table 3. Comparison between antibiotic susceptibilities (as measured by zone of inhibition in mm) of MDR clinical isolates of *P. aeruginosa* and the same isolate after adaptation to 2-PE.

Antibiotic	Zone of inhibition (mm) (±SD)		
	MDR *P. aeruginosa*	2-PE resistant MDR *P. aeruginosa*	*P-value
Gentamicin	2.3 (0.4)	25.3(.4)	p<0.05
Amoxacillin	0(0)	36(.6)	p<0.05
Doxycyclin	3.3(0.57)	30.3(.35)	p<0.05
Amikacin	21.6(0.15)	23.6(.4)	ns∗
Rifambicin	0(0)	27.6(.61)	p<0.05
Norfloxacin	0(0)	28(.6)	p<0.05
Vancomycin	0(0)	21.8(.58)	p<0.05
Cefotaxime	0(0)	23.6(.55)	p<0.05
Cephalexin	0(0)	29(.45)	p<0.05
Ofoxacin	0(0)	24(.17)	p<0.05
Chloramphenicol	2.3(.40)	25(.05)	p<0.05
Tobramycin	13.6(.46)	20(.88)	ns
Azithromycin	25(.26)	18.3(.47)	ns
Sulphamethoxazole	0(0)	27.2(.17)	p<0.05
Ticar/clav	0(0)	35.3(.66)	p<0.05
Ciprofloxacin	0(0)	28.3(.60)	p<0.05
Cefixim	0(0)	15(1.21)	p<0.05
Minocyclin	9.6(.46)	27(.60)	p<0.05
Aztreonam	18.6(.15)	0(0)	p<0.05
Ceftizoxime	13.3(1.15)	28(.39)	ns
Piperacillin	17(.78)	34(.85)	p<0.05
Ceftazidime	15(.1)	19.3(.84)	p<0.05
Ampicillin	0(0)	34.3(.40)	p<0.05
Imipenem	0(0)	39.5(.35)	p<0.05
Nitrofuranton	0(0)	21(.1)	p<0.05

*P value calculated using a non-parametric Mann-Whitney U test; ∗ns: not significant.

was completely lost ($p<0.005$) (Table3). Such alterations in antibiotic sensitivity have been documented previously (Joynson et al., 2002), though MDR isolates were not the focus of such studies.

2-PE adaptation by *P.aeruginosa* PAO1 was accompanied by an increased sensitivity towards the antibiotic aztreonam (Abdel Malek and Badran, 2010). Interestingly though, adaptation of MDR *P. aeruginosa* isolates to 2-PE resulted in aztreonam resistance. Aztreonam resistance is known to be mainly due to increased *mexA* expression (Walsh et al., 2003). However, Abdelmalek and Badran (2010) found that the antibiotic susceptibility changes in the 2-PE resistant *P. aeruginosa* PAO1 are independent of RND efflux pumps. Similarly, we can say that the gain of the aztreonam resistant phenotype in our case might not be due to over expression of *mex A*. This poses yet another question into the complex arena of biocide/antibiotic cross resistance. Two very clinically important and useful antipseudomonal antibiotics, imipenem and gentamycin were not affected by 2-PE adaptation in the PAO1 strain (Abdel Malek and Badran, 2010) however, MDR *P. aeruginosa* gained full sensitivity to these two antibiotics after the adaptation to 2-PE in this study (Table 3). The 2-PE adaptation of MDR *P.aeruginosa* strain was shown to manifest differently than the laboratory strain PAO1 *P. aeruginosa*. Although both involved increased susceptibility to antibiotics and reduced to others but the response to aztreonam and imipenem was different in both strains.

Imipenem resistance is known to be due to suppression of OprD thus regaining sensitivity to imipenem by the MDR 2-PE strain and the loss of aztreonam susceptibility further supports our hypothesis that the reason behind these changes is mainly outer membrane alterations. 2-PE has been reported to induce outer membrane changes (Abdel Malek and Badran, 2010; Denyer, 1995) which according to our results varies between species. MDR isolates of *P. aeruginosa* are known to overexpress and express multiple efflux pumps, as well as undergo changes in outer membrane structure (Toma´s et al., 2010) thus the effect of 2-PE will eventually express a different phenotype than the wild type PAO1.

Does antibiotic resistance bring about biocide resistance? A question raised by Stickler (2002) who examined

clinical isolates that were chlorhexidine resistant as well as antibiotic resistant from patients suffering from urinary tract infection (UTI). The author explained that the emergence of such phenotype is due to excessive use of chlorhexidine in catheter management. In the present study, clinical MDR strain of *P. aeruginosa* had a significantly ($p<0.05$) elevated MIC towards 2-PE in comparison to the PAO1 strain (Table 1) although the MDR clinical strain was not exposed to 2-PE like in the case of chlorhexidine. Thus, there has been a low level of resistance towards this biocide that accompanied the MDR status that we would loosely say it came with the antibiotic resistance. The present research is still in the pursuit of understanding the relationship between biocide misuse and antibiotic resistance. Results obtained in this research draw the attention to a different, rather interesting, effect that can be imposed on antibiotics susceptibility as a result of biocide resistance. These findings suggest further questions that need more investigation. For example, what exactly in 2-PE adaptation triggered this effect? Is this effect on multidrug resistance uniform for other biocides? We believe that these findings can open up a new window in the future for the treatment or prevention of multidrug resistant nosocomial infections.

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