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The Effect of Triclosan Adaptation on Antimicrobial Resistance among Clinical *Escherichia coli* Isolates from Egyptian Patients

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Abstract

There is a possible link between exposure to Triclosan (TCS) and changes in antimicrobial susceptibility. The change in the tolerance of clinical *Escherichia coli* (n=45) isolates to the biocide TCS, changes in antibiotic resistance and differences in the efflux pump mechanism were analyzed. 45 *E. coli* isolates were obtained. The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) of TCS, and the expression of four efflux pump encoding genes in antibiotic resistant isolates were determined before and after TCS adaptation. The number of TCS-tolerant isolates was 11 (24.4%). After adaptation, the percentage of tolerant isolates increased to 42.2% (n=19). A significant change (p<0.05) in antimicrobial resistance of the tested isolates (n=45) before and after TCS adaptation was detected for ceftazidime, ceftriaxone, ertapenem, imipenem, amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin and doxycycline. Among the new TCS tolerant isolates (n=8). there was an increase in TCS MIC as well as the MBC after TSC adaptation. The adapted isolates exhibited a significant increase in the expression of *mdfA* and *norE* genes (p=<0.001). There is a strong correlation between efflux pump gene overexpression and susceptibility to TCS and other antimicrobials.

Keywords: Triclosan, Efflux pump, MIC, Cross-resistance, *Escherichia coli*

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INTRODUCTION

Triclosan (TCS) (2,4,4-trichloro-2-hydroxy-diphenyl ether) is a broad spectrum non-antibiotic antimicrobial agent that is a common ingredient in more than 200 consumer products, such as detergents, soaps, disinfectants, toothpaste, and shower gels.[1] TCS is characterized by a wide activity range against several microorganisms, for example, *Staphylococcus aureus* and *Escherichia coli*.[2,3]

TCS, like other biocides, is not used to treat host infections, and as a result, the regulations on use of this compound are not as strict as they are for antibiotics. Biocides are widely used without restrictions in many fields, such as the food industry, household hand-cleaning chemicals, and dental washing. Biocides may select for antibiotic-resistant microorganisms.[4] Even at low concentrations, TCS has been shown to cause antibiotic resistance via different mechanisms.[4,5]

Previous studies investigated the potential link between clinical isolates exposed to biocides, such as TCS, and altered antimicrobial sensitivity and indicated that repeated exposure to sub-lethal concentrations of biocides can contribute to the spread of antimicrobial resistance. Studies on *Staphylococcus aureus* and *E. coli* showed that tetracycline and chloramphenicol resistance was elevated tenfold after exposure to TCS.[2,6] TCS is assumed to exert pressure on the bacterial community, conferring bacterial resistance to the microorganisms, raising concerns about the possibility of cross-resistance between antimicrobials or antibiotics.[7,8]

Several mechanisms appear to drive antimicrobial resistance after the adaptation of TCS. The overproduction of efflux pumps is regarded as one of the most prevalent biocides resistance mechanisms.[8] Overproduction of efflux pumps is associated with low-level resistance to biocides, and several classes of antibiotics, such as fluoroquinolones, β-lactams, tetracyclines and macrolides.[9] Efflux pumps enable bacteria to physically remove an ingredient from the intracellular space by pumping it through the membrane back into the surrounding environment. This mechanism has proved to be effective against several antimicrobials as well as biocides such as TCS.[9,10] Examples of these efflux pumps are the resistance nodulation division (RND) family, the staphylococcal multi-resistance (SMR) family and the significant facilitator super (MFS) family.[11, 12]

Among the systems investigated for biocide extrusion, TolC, AcrAB, TolC AcrEF and EmrE from *E. coli*[13-15] and MexCD-OprJ, MexAB-OprM, as well as MexEF-OprN from *P. aeruginosa*[16] are the most highlighted. The majority of non-specific efflux pumps can remove antibiotics from an intracellular location and thus cause resistance. Therefore, when the bacteria acquire a non-specific efflux pump via horizontal gene transfer after TCS exposure, these bacteria frequently develop antibiotic resistance as well.[17,18]

As several biosides are used nowadays, the risk of inappropriate use of these biosides can lead to the spread of antibiotic-resistant bacteria. For this reason, more research is needed to further investigate this topic. In particular, studies on the possible role of biocides in the development of antibiotic resistance with an Egyptian setting are un-common. This study seeks to address this point.

PATIENTS AND METHODS

The current study was conducted in the Department of Medical Microbiology and Immunology in the Central Laboratory, Faculty of Medicine, Menoufia University.

Bacterial isolation and antimicrobial susceptibility

45 *E. coli* isolates were obtained from Menoufia University hospitals from March 2019 to March 2020. Each isolate was obtained from a patient suffering from an infection, such as a respiratory tract or urinary tract infection or bacteremia. Samples were cultured on nutrient, blood, and MacConkey agar media. Plates were incubated at 37°C for 24 h. Identification was done using selective media and conventional biochemical methods as described by El-Hadedy and El-Nour.[19] Isolates were stored in tryptic soy broth supplemented with 16% glycerol and frozen at -80°C. The study was approved by the local ethics council at the Faculty of Medicine Menoufia University, and verbal consent was obtained from each patient before sampling.

Antimicrobial susceptibility testing was conducted using agar dilution, along with adherence to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2009).[20]

Detecting the minimum inhibitory TCS concentration (MIC) as well as minimum bactericidal concentration (MBC) via the method of broth dilution

The TCS MIC for each of tested isolates was determined using broth microdilution method using a microtitration plate according to the CLSI guidelines.[20] In each plate had a growth control well (postive control) and uninoculated well as (negative control). the plates were incubated for 24 h at 37°C. The lowest concentration of antibiotic that completely inhibited the bacterial growth as indicated by no visible turbidity in the well in comparison with the positive and negative controls was accepted as the MIC.

After determination of the MIC, the wells that showed growth inhibion were subcultered onto Mueller-Hinton (MH) agar plates to determine the minimum bactericidal concentration (MBC). The MBC was defined as the lowest concentration of TCS required to kill the bacteria after culture and incubation 24 h at 37◦C. All of these determinations was carried out in triplicate According to Curiao et al.,[21] isolates were considered tolerant if the TCS MIC was higher than 7.5 µg/ml-1

Adaption to an increased TCS concentration

The adaptive isolation response to TCS was tested by daily exposure to gradually increasing sub-lethal concentrations of TCS for seven days according to the method described by Soumet et al.[22]

The experiment started with concentration of 0.5× MBC of TCS in MH broth which was incubated for 24 h at 37°C. Once bacterial growth was detected, 100 µl of the bacterial suspension was transferred to fresh MHB (10 ml) supplemented with a higher TCS concentration (concentrations used ranged from 1 to 10 µg/ml and increased by 1 µg /ml per day). This procedure continues until no growth is detected after incubation for 24 h at 37°C. When no growth is detected, the previous concentration is used as the endpoint.

The bacteria were spread from the last tube with the recorded bacterial growth, with a loop (10 µl) on MH agar, and incubated for 24 h at 37°C to confirm growth as well as to allow storage. As a control, a bacterial suspension (100 µl) was added to MH broth (10 ml) and the process above used but in the absence of TCS.

Determining efflux pump genes by quantitative Reverse Transcription PCR (RT-qPCR)

Quantitative reverse transcription PCR assays for the efflux pumps encoded via the *mdfA, acrB, norE*, and *yihV* genes were investigated using the primers previously recommended by Huguet et al.[23]

RNA was extracted utilizing the RNeasy® Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions with minor modifications. The bacterial cells were disrupted using lysosome (Sigma-AIdrich) as well as proteinase K (Qiagen). RNA purification using the RNase-Free DNase Set Kit (Qiagen) was performed to remove any residual DNA. The quantity and quality of the extracted RNA were measured by a Nanodrop spectrophotometer (Nanodrop Technologies).

Afterwards, the RNA was transcribed into cDNA via a cDNA power synthesis kit (First Strand cDNA Synthesis Kit) (Thermofisher Scientific, Applied Biosystems, USA), as recommended by the manufacturer. The synthesized cDNA was used as a real-time PCR template, utilizing SYBR Green II master blend (QuantiTect SYBR Green PCR Kit, Applied Biosystems, USA) in Applied Biosystems 7500, software version 2.0.1. (Applied Biosystems, USA).

Quantitative PCR was carried out on a Chromo4™ Real-Time Detector (Bio-Rad, Marnes-

Table 1. Primers and annealing temperatures utilized in qRT-PCR

Gene	Forward	Reverse	Annealing temp.
acrB	5/-GAAGAGCACGCACCACTACAC-3	5/-GCAGACGCACGAACAGATAGG-3	55
mdfA	5/-TTTATGCTTTCGGTATTGG-3/	5/-GAGATTAAACAGTCCGTTGC-3/	52
norE	5/- TCGCAGGACATCAGATTG-3/	5/-CAGACACCCACCATAAGC-3/	55
yihV	5/-GGCTATCATCCTCGTCTTCC-3/	5/-GCGTCATCCACCAGTAACC-3/	54
gapA	5/-GGACGAAGTTGGTGTTGAC-3/	5/-TTCTGAGTAGCGGTAGTAGC-3	54

la-Coquette, France) for the four efflux pump genes *mdfA, acrB, norE*, and *yihV.* The gapA gene was employed as a housekeeping gene(as it did not exhibit any significant variation in expression among the samples) to normalize the levels of gene expression of a*crB, mdfA, norE* and *yihV.* For each gene, the reactions were performed in duplicate. The total volume of the reaction was 20 µl containing 10 µl Power SYBR® GREEN PCR Master Mix 5 µl cDNA, 1 µl of each primer (0•3 µmol l−1 final concentration) and 3 µl sterile water. After an initial step (94°C for 7 min), the thermal cycling protocol was as follows: 40 cycles of PCR for 15 s at 95°C for denaturation, for 15 s at 52 or 55°C for annealing, and for 15 s at 72°C for extension. Data were analyesd as performed by Huguet et al.[23] The primers that were used in the current study are displayed in Table 1.

The qRT-PCR efficiencies were calculated from the slope of a linear regression model, for each pair of primers when the reaction efficiency is estimated at (E) = 10(−1 / slope).[24,25] For serial concentrations of cDNA, the Ct calculated the calibration curve. The rotor gene Q v. 2.3.1 program (QIAGEN-Germany) was employed for the interpretation of the findings. The relative expressions of *acrB, mdfA, norE,* and *yihV* were completed through a comparative Ct method,[26] in which the total target genes are normalized to a housekeeping reference gene (*gapA*). Each test was performed using a melting curve analysis to validate the accuracy of amplification and the absence of primer dimers.

Statistical analysis

Statistical analysis was conducted by SPSS, version 20; SPSS Inc., Chicago, Illinois, USA. Data were recorded, tabulated, and analyzed using Excel software for Windows. The susceptible and resistant strain distributions, before the following adaptation have been compared using the Chi-square test. A p-value < 0.05 was considered to be statistically significant.

RESULTS

A total of 45 *E. coli* isolates were obtained. The MIC values for TSC varied from 1 to 64 µg/ml. Moreover, among these *E. coli* isolates, 24.4% (n=11) were tolerant to TCS (Table 2). After adaptation, another eight (17.7%) isolates (named as E1 to E8), became tolerant to TCS. The total number of tolerant isolates became 19 (42.2%).

Table 2. Minimum inhibitory concentration (MIC) of TCS for the 45 *E. coli* isolates

TCS Concen. (µg/ ml)	TCS (MIC)	
	Before adaptation No (%)	After adaptation No (%)
1	4 (8.9)	1 (2.2)
2	13 (28.9)	10 (22.2)
4	17 (37.8)	15 (33.3)
8	2 (4.4)	2 (4.4)
16	7 (15.6)	14 (31.1)
32	2 (4.4)	3(6.7)
64	0	0

Table 3. Minimum inhibitory TCS concentration (MIC) and minimum bactericidal concentration (MBC) of tolerant *E. coli* isolates after adaptation (N=8)

Triclosan resistant isolates after adaptation (n= 8)	TCS MIC (µg/ml)		TCS MBC (µg/ml)	
	Before adaptation	After adaptation	Before adaptation	After adaptation
E1	4	16	4	32
E2	2	16	4	32
E3	8	32	8	32
E4	4	16	4	16
E5	2	16	4	16
E6	4	32	4	64
E7	4	16	4	32
E8	4	16	4	32

MIC (minimum inhibitory concentration), MBC (minimum bactericidal concentration).

Among the eight TCS tolerant *E. coli* isolates after adaptation, there was an increase in TCS MIC as well as the MBC (Table 3).

A significant change ($p<0.05$) in antimicrobial resistance of the tested isolates (n=45) before and after TCS adaptation was detected for ceftazidime, ceftriaxone, ertapenem, imipenem, amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin and doxycycline.

Table 4. Antimicrobial susceptibility among 45 *E. coli* isolates, before and after TCS adaptation

Antimicrobial agents	Before TCS adaptation Resistant		After TCS adaptation Resistant		P-value	Relative change
	No	%	No	%		
amoxicillin/clavulanic acid	42	93.3	44	97.8	>0.05	4.5%
piperacillin/tazobactam	38	84.4	42	93.3	>0.05	9.2%
cefoxitin	43	95.6	44	97.8	>0.05	2.2%
ceftazidime	34	75.6	41	91.1	*<0.05	15.5%
ceftriaxone	35	77.8	42	93.3	*<0.05	15.5%
cefepime	42	93.3	43	95.6	>0.05	2.3%
ertapenem	15	33.3	26	57.8	*<0.05	24.5%
imipenem	17	37.8	29	64.4	*<0.05	26.6%
meropenem	22	48.9	31	68.9	>0.05	20%
amikacin	31	68.9	40	88.9	*<0.05	20%
gentamicin	28	62.8	37	82.2	*<0.05	19.4%
tobramycin	26	57.8	36	80	*<0.05	22.2%
ciprofloxacin	32	71.1	42	93.3	*<0.05	22.2%
levofloxacin	24	53.3	33	73.3	*<0.05	20%
doxycycline	22	48.9	32	71.1	*<0.05	22.2%

* $p<0.05$, statistically significant.

Before the adaptation to TCS, the highest resistance level was recorded to cefoxitin (95.6%) followed by amoxicillin/clavulanic acid (93.3%), and finally cefepime (93.3%). After adaptation, the resistance increased to 97.8%, 97.8%, and 95.6%, respectively (Table 4).

Table 6 shows that there was a significant elevation in the efflux pump gene expression for *mdfA* and *norE* in the TCS-adapted isolates ($p<0.001$)

Table 5. Analysis of the relative gene expression of *E. coli* (n=8) after TCS adaptation

Isolates	Relative gene expression							
	acrB		*mdfA*		*norE*		*yihV*	
	Before adaptation	After adaptation	Before adaptation	After adaptation	Before adaptation	After adaptation	Before adaptation	After adaptation
E1	1	1	2.874	4.602	401.7	576.929	1.115	1.589
E2	1.076	1.114	2.953	6.055	1	1	0.985	1.343
E3	1	1.045	1.763	3.937	539.65	843.768	1.003	1.449
E4	1.194	1.706	2.985	7.626	63.9	63.9	1.728	2.098
E5	1	1.081	1	1	12.935	15.579	1.554	1.793
E6	1.076	1.259	3.919	7.495	435.879	943.321	1.025	1.128
E7	1.093	1.263	4.642	8.793	328.459	706.77	1	1.057
E8	1.125	1.558	5.829	7.307	679.51	984.418	0.659	1

Table 6. Analysis of the relative gene expression of *E. coli* (n=8) after TCS adaptation

Gene	qRT-PCRFold Change [std error]	p-value
acrB	1.665 [1.512 to 1.814]	0.024
mdfA	2.426 [2.275 to 2.573]	<0.001*
norE	2.124 [1.743 to 1.915]	<0.001*
yihV	1.204 [1.432 to 1.715]	0.04

* statistically significant

DISCUSSION

The role of biocides in the selection and the prevalence of antibiotic resistance has emerged recently. Therefore, several studies have focused on investigating the biocide susceptibility of clinical bacterial isolates.[27,28] One of the most fundamental methods causing antimicrobial resistance is the exposure of bacteria to sub-inhibitory antibiotic concentrations. Furthermore, bacteria tend to follow a similar path during adaption to biocides at sublethal concentrations.[29-31]

There is a growing concern that excessive use of TCS is the reason for the accelerated emergence of TCS tolerance among clinical isolates. Consequently, the existence of TCS-tolerant *E. coli, S. aureus, Klebsiella pneumoniae,* and *Acinetobacter baumannii* has been reported.[32,33]

Initially, eleven (24.4%) of the 45 isolates showed an elevated MIC to TCS. After adaptation, the percentage of TCS tolerant isolates increased to 42.2% (n=19) (Table 2).

A significant change ($p<0.05$) in antimicrobial resistance of the tested isolates (N=45) before and after TCS adaptation was detected for ceftazidime, ceftriaxone, ertapenem, imipenem, amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin and doxycycline (Table 4). Sonbol et al.[34] in his study reported a similar pattern of a significant increase in antibiotic resistance after adaptation to TCS, except that we did not detect a rise in the MIC values for amikacin and sulphamethoxazole/trimethoprim.

Many studies have examined the potential link between exposure of clinical isolates to biocides and the development of biocide resistance and any associated change in antimicrobial susceptibility. Romanova et al.[35] found that biocide-adapted *Listeria monocytogenes* had a 2 to 4-fold elevation in MIC to gentamycin, kanamycin and novobiocin as compared with the wild type. Karmakar et al.[1] reported a four-fold increase in MIC and MBC concentration of gatifloxacin among *Aeromonas hydrophila* and *Edwardsiella tarda* isolates after exposure to TCS. Other studies investigated a decrease in antibiotic susceptibility in *E. coli*, *Ps. aeruginosa, Salmonella,* and *S. aureus* after exposure to biocide or adaptation.[36-40] These findings indicated that exposure to biocides could alter antibiotic susceptibility.

In *E. coli*, fluoroquinolone resistance occurs through several mechanisms - one being changes in the outer membrane porins (OMPs). These changes may be associated with overexpression of an efflux pump gene and can lead to reduced susceptibility to fluoroquinolones.[41] The MFS, multi-drug, toxic compound extrusion (MATE), and RND families are chromosome-dependent efflux systems, which are the main cause of resistance to fluoroquinolones.[42] OmpC and OmpF from the porin family are examples of OMPs and efflux pumps that contribute to fluoroquinolone resistance.[43] AcrB, AcrF, and YhiV are RND family elements, while NorE is an example of the MATE family, and MdfA is from the MFS efflux pump family.[43]

In a study done by Zeng et al.[11] on *E. coli* isolated from urine an increase in the overexpression of *yihV, acrB, acrD,* and *mdfA*, all belonging to the MFS family was noted. Romanova et al.[35] reported that the increase in MIC of biocides in adapted *L. monocytogenes* strains was due to the increased expression of efflux pump-encoding gene mdrL. In another study conducted by Curiao et al.[21] exposure to the biocide TCS resulted in cross-resistance to antimicrobials with overexpression of efflux pump gene regulators. Similar results of an increase in the expression of efflux gene pump were reported among biocide adapted *E. coli*, *Serratia marcescens* and *Acinetobacter baumannii* isolates.[44-46]

In brief, our results showed that repeated exposure to sub-lethal concentrations of TCS increased the expression of efflux genes which resulted in an altered bacterial susceptibility to antimicrobial compounds

CONCLUSION

We concluded that stepwise exposure to sublethal concentration of TCS can be responsible for adaptive expesssion of mechanisms that affect efflux activity which in turn influences the bacterial susceptibility to different antimicrobials. More studies are needed to study the molecular mechanisms that are responsible for the increase in antimicrobial resistance among the biocide adapted *E. coli* isolates. As TCS disinfectants are used in uncontrolled way in Egyptian hospitals and may be a contributing factor for increasing antimicrobial resistance. A well planned longitudinal study is recommended to investigate the molecular changes before and after repeated exposure to the biocide TCS.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The study was approved by the local ethics committee at the Faculty of Medicine, Menoufia University, Egypt.

INFORMED CONSENT

Informed consent was taken from each patient before the study.

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