Overexpression of target enzyme gene \textit{fabl} and efflux pump decrease triclosan susceptibility in \textit{Escherichia coli}

\textbf{CURRENT STATUS: UNDER REVIEW}

\textit{BMC Microbiology}  \textit{BMC Series}

Weiliang Zeng  
Wenzhou Medical University

Wenyia Xu  
Wenzhou Medical University First Affiliated Hospital

Ye Xu  
Wenzhou Medical University First Affiliated Hospital

Wenli Liao  
Wenzhou Medical University First Affiliated Hospital

Yajie Zhao  
Wenzhou Medical University First Affiliated Hospital

Xiangkun Zheng  
Wenzhou Medical University

Chunquan Xu  
Wenzhou Medical University First Affiliated Hospital

Tieli Zhou  
Wenzhou Medical University First Affiliated Hospital

Jianming Cao  wzcjmimg@163.com  
Wenzhou Medical University  
Corresponding Author

\textbf{DOI:}  
10.21203/rs.2.21275/v1

\textbf{SUBJECT AREAS}  
\textit{Applied & Industrial Microbiology}  \textit{General Microbiology}

\textbf{KEYWORDS}  
\textit{Escherichia coli, triclosan, efflux pump, resistance, fabl}
Abstract

**Background:** *Escherichia coli* isolates, the most opportunistic pathogen in the gut, are responsible for the most acquired infections. Triclosan is an effective disinfectant for inhibits microorganisms, but its widespread use causes its residue in urine, resulting in long-term exposure of *E. coli* in the intestine to triclosan environment and increasing triclosan resistance. We aim to provide the mechanism of action of *E. coli* isolates against triclosan and the molecular epidemiological analysis of triclosan-resistant strains.

**Results:** Five triclosan-resistant isolates were screened out from 200 *E. coli* isolates by agar dilution method by to further study, interestingly, multidrug-resistant and cross-resistance phenotypes were observed in triclosan-resistant strains, but not in susceptible strains, and all except one exhibited an inhibition of efflux pump activity by efflux pump inhibition testing. Furthermore, compared with susceptible *E. coli* strain ATCC 25922, except fabI, increased expression were also found in efflux pump encoding genes ydcV, ydcU, ydcS, ydcT, cysP, yihV, acrB, acrD and mdfA in studied strains which had different PFGE patterns and STs types.

**Conclusions:** These findings indicated that triclosan resistance in *E. coli* were mainly involved by overexpression of fabI gene, and there was a close association between overexpression of efflux pump with reducing susceptibility to triclosan. Besides, we described cross-resistance between triclosan and antibiotics may be related to the exposure time of triclosan.

**Background**

*Escherichia coli* isolates, one of the common opportunistic pathogens, are responsible for the most hospital and community acquired infections, such as a variety of intestinal and extraintestinal infections [1]. They are considered to be one of the leading causes of
urinary tract infection, as well as serious infections in the immunocompromised patients [2, 3]. Over the past few decades, self-medication and antibiotics misuse led to the increasing resistance in clinical practice. Even worse, some of E. coli isolates have been reported as multidrug-resistant (MDR) pathogens (generally considered non-susceptible to ≥ 3 antibiotic classes) [4, 5], which makes the clinical treatments of infections caused by E. coli face a great challenge.

Triclosan, a broad-spectrum and highly effective antibacterial agent, can inhibit various microorganisms at low concentrations, and be bactericidal at high concentrations [6]. In fact, it is not only used in disinfections, but also in medical equipment to prevent infections. According to a recent research report, there has a clinical effectiveness of triclosan-coated sutures compared to uncoated sutures for surgical site infections (SSIs) prevention [7]. Hence, triclosan plays a key role in reducing the dissemination and spread of pathogenic bacteria in hospital and community environments. Unfortunately, owing to increased clinical use, obvious levels of triclosan in various natural and engineered environment, such as soil and water, even in body fluids, such as urine, have been reported [8, 9]. In China, Yin et al. estimated the average concentration of triclosan reached 0.36 μg/l in 80% urine samples [10]. Furthermore, triclosan was known as a “new environmental endocrine disruptor” due to its potential endocrine disrupting effects, which took an adverse effect on human health [11]. Therefore, widespread use or sustained exposure of triclosan has given rise to concern regarding its impact on accelerating the emergence of MDR E. coli, which poses great threats to public health as well as put the people at higher risk [12].

Although triclosan has a benefic effect on sterilizing for most pathogens in hospital settings, as previously mentioned, long-term exposure to triclosan have promoted a reduced sensitivity of triclosan in E. coli through extensive resistance mechanisms [13]. Of
these resistance mechanisms, active efflux, reducing the drugs concentration in the bacteria whereby efflux pump systems to pump the intracellular antibacterial drugs out of the cell, conferred bacteria the ability to against a wide range of antimicrobials and biocides, including triclosan [14, 15]. Indeed, many drug efflux pumps that were known to mediate resistance to traditional antibiotics and biocides include the efflux systems the resistance nodulation division (RND) family, the major facilitator superfamily (MFS), the staphylococcal multi-resistance (SMR) and multidrug and toxic compound extrusion (MATE) families [16]. Additionally, triclosan was an inhibitor of enoyl-acyl carrier protein (ACP) reductase (FabI) enzyme, which involved in lipid biosynthesis and encoded by fabI gene. Mutations within fabI contribute to E. coli resistance to triclosan [17, 18]. However, the role of many efflux pumps in triclosan resistance in E. coli is not well understood. In order to provide a better scientific theoretical basis for the clinical rational use and nosocomial infections control in the future, investigations are urgently warranted.

In this study, we focus on gene expression and molecular typing to establish relation between the expression levels of efflux pump and triclosan resistance, and to investigate transmission of drug resistance in E. coli.

Results

Susceptibility of triclosan and antibiotics before and after exposure to triclosan

MICs of triclosan among 200 isolates were ranging from 0.03125µg/ml to 8 µg/ml, five triclosan-resistant isolates were screened out (2.5%, 1/200), as shown in Table 1. Interestingly, triclosan-resistant E. coli isolates tend to be resistant to multiple antibacterial agents, including ampicillin, cefepime, ceftazidime and gentamycin, but randomly selected five triclosan-sensitive strains did not exhibit multidrug-resistant phenotypes. Whether triclosan affects antibiotics sensitivity, further serial passage experiments were needed and performed.
Stable triclosan-mutant strains were generated by continuous passage of triclosan-
susceptible isolates DC8361, DC8363, DC8400, DC8413 and DC8510. After exposure to
subinhibitory triclosan concentration only four days, the triclosan MICs increased to 1, 16,
1, 4 and 1μg/ml respectively, inversely, the MICs values of tested antibiotics were no
change (Tables 1).

**Efflux pump phenotype testing**

We sought to further investigate the basis for elevated triclosan MICs among the resistant
isolates. The triclosan MICs of DC8358, DC8419, DC8424 and DC8724 reduced by 8, 4, 4
and 16 times in the presence of the concentration of 10 μg/ml of CCCP compared to the
absence of CCCP, respectively. The result indicated that efflux pump systems were
extremely active among above mentioned four isolates. Inversely, DC8603, unlike other
resistant strains, showed a negative phenotype in efflux pump testing, which the MICs
value of triclosan had not changed whether with the presence or the absence of CCCP
(Table 2).

**Analysis of fabI gene mutation**

PCR revealed that fabI and 14 efflux pump encoding genes were present in all tested
strains except acrF gene. A variety of different mutations were detected in both resistant
and susceptible strains. In DC8419 and DC8424, the Gly79Ala and Ala69Thr mutations
were found, respectively. However, these two mutations were also observed in susceptible
strains. Besides, we discovered other mutations of fabI gene in susceptible strains, such
as Met2Arg, Ser5Leu, Val4Ser and Asp235Glu. Nonsense mutations were found in other
resistant strains (Table 1).

**Expression levels of target encoding gene and efflux pump encoding genes**

The expression levels of fabI gene were evaluated in this study. Increased expression (> 2-fold) of fabI gene was observed in all triclosan-resistant strains. Compared with the
triclosan-susceptible control strain ATCC 25922, the fold-changes of *fabI* gene with notable changes in the expression levels were between 5.69 to 41.85 times (Fig. 1).

In addition, to gain the better understanding of the relationship between triclosan resistance and gene expression level of efflux pump, expression of different efflux pump types was also examined for resistant strains as shown in Fig. 2 and Tables 2. Compared to *E. coli* ATCC 25922, the expression of *ydcV* was increased obviously (> 2-fold) in DC8358 (fold-changes: *ydcV*, 5.71 ± 0.68). Exhibited enhanced expression of *ydcV*, *yihV* and *acrB* (fold-changes: *ydcV*, 8.74 ± 0.61; *yihV*, 3.57 ± 0.52; *acrB*, 3.44 ± 0.21, respectively) was found in DC8419. For DC8424, the expression of *ydcU*, *ydcS*, *yihV*, *acrD*, and *mdfA* (fold-changes: *ydcU*, 4.71 ± 0.13; *ydcS*, 2.8 ± 0.42; *yihV*, 6.82 ± 0.65; *acrD*, 2.63 ± 0.14; *mdfA*, 5.13 ± 0.26, respectively) was increased. Upregulation of active efflux pump gene *ydcT*, *ydcU*, *ydcS*, *cysP*, and *yihV* (fold-changes: *ydcT*, 6.56 ± 0.56; *ydcU*, 18.25 ± 1.36; *ydcS*, 8.76 ± 0.49; *cysP*, 3.89 ± 0.2; *yihV*, 2.00 ± 0.03, respectively) was observed in DC8724. The result was consistent with efflux pump inhibition testing, which indicated that overactivity of efflux pump induced overexpression of efflux pump gene, which in turn mediates the susceptibility of triclosan.

**Molecular epidemiological analysis**

PFGE analysis revealed that the similarity of these isolates was low (< 0.85) due to the large differences in PFGE patterns. Similarly, the results of MLST confirmed that they were categorised into multiple and scattered STs, including ST3, ST833, ST567, ST471 and ST1, respectively (Fig. 3). In short, the results above illustrated that triclosan-resistant strains had extremely low clonal relatedness in this study.

**Discussion**

In our study, 200 *E. coli* isolates were collected from patients with UTIs, and only five triclosan-resistant strains (2.5%, 5/200) were selected, the rate of which was lower than
the previous report [19]. These triclosan-resistant strains, rather than susceptible strains, were characterized by a MDR profiles, we supposed that there may be a cross-resistance between triclosan and antibiotics in *E. coli* based on previous studies [20, 21]. To test our hypothesis, DC8361, DC8363, DC8400, DC8413 and DC8510 parent strains that were sensitive to almost all antibiotics and triclosan, have been randomly selected for serial passage experiments. But it was not consistent with our supposition that changes in antimicrobial susceptibility were not observed before and after these strains adaptation, except triclosan. Actually, cross-resistance has been reported under the long-term selective pressure of triclosan, rather than in the short term according to Kampf et al. [20, 22], that's why cross-resistance has not been observed during a continuous induction period of four days. Based on the obtained phenomena, there maybe had a close relationship between cross-resistance and exposure time of triclosan, which provided a sufficient evidence for clinical practice to avoid long-term use of triclosan to prevent cross-resistance. Moreover, it is worth noting that significantly reduced triclosan sensitivity was found by serial passage experiments, which provided a beneficial guidance for reasonable use of triclosan, including concentration, dosage and exposure time, in order to prevent the increase resistance of pathogens to triclosan.

As shown in previous studies, *fabI* gene mutations such as Gly93Val, Leu94Phe, Met159Thr, Ala197Gly, and Phe203Leu were reported in triclosan-resistant *E. coli* [14, 15]. Nevertheless, these mutations were not identified in our study. Notably, it was the first time that two different mutations in *fabI*, Gly79Ala and Ala69Thr were characterized. However, these mutations were detected both in resistant strains and in susceptible strains, in other words, they may have no effect on the susceptibility of triclosan. Besides, as described before [23], overproduction of target gene *fabI* was also observed, which was the most commonly described resistant mechanisms of triclosan in *E. coli*. 
Previously study suggested effectively upregulated efflux pump genes played an important role in triclosan non-susceptibility *E. coli* in a hostile environment [24]. In this respect, we tested the activity of four different types of Tolc and their relative expression of the corresponding coding genes, including ABC transporters system, Arac-regulator genes, the MdfA efflux Tolc, and the NorE efflux pump. Under triclosan stimulation, increased efflux pump activity of the isolates were found in our study, and the expression levels of ABC transporters system encoding genes *ydcT, ydcU, ydcV, ydcS*, and *cysU* and RND-type tolc encoding genes *acrB, acrD* and *yihV*, as well as *mdfA* gene which belonging to MFS family had a significant increase compared with ATCC 25922, which were consistent with a previous study in China [23]. In contrast to previous studies we did not observed any increase in the expression levels of other efflux pump encoding genes as evidenced by Sonbol and Curiao et al. [15, 21], maybe it can be understood that ABC transport efflux pump, RND-type tolc and MFS family activity a stronger advantage than others during making adaptive changes of the studied isolates to triclosan [15, 21]. All in all, we found that there was a strong relationship between gene overexpression and the increased tolerance of *E. coli* against triclosan, which suggested multiple efflux pumps may synergistically mediate triclosan resistance.

In addition, PFGE and MLST were useful and helpful methods for phylogenetic relationships of a large collection of bacterial lineages. Different pulse types and STs types were observed in the studied isolates by PFGE and MLST, suggesting that there was no transmission and a clonal dissemination among these triclosan-resistant strains. However, our study also has some limitations. Although, cross-resistance between triclosan and antibiotics was noticed here, we did not illuminate the possible triclosan cross-resistance mechanisms, which is the focus of our future research.

Conclusions
Our study fills the gap in the field that various efflux pumps mediated triclosan resistance by analyzing genes expression which indicated that triclosan resistance was mainly mediated by fabI overexpression, and corresponding overexpression of efflux pump genes may contribute to enhanced triclosan tolerance in E. coli. Besides, we found cross-resistance between triclosan and antibiotics may be related to the exposure time of triclosan, which will provide a better scientific theoretical basis for rational use of triclosan in clinic and nosocomial infections control. Hence, the necessary monitoring methods, such as MICs, β-lactamase and resistance genes mutation, are still important for clinical control of triclosan resistance.

Methods

Bacterial strains and identification

A total of 200 uropathogenic non-repetitive E. coli strains were obtained from the patients with urinary tract infections from Affiliated Hospital of Wenzhou Medical University in 2018. All bacteria were identified by the Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS; bioMérieux, Lyons, France).

Antimicrobial susceptibility testing

Agar dilution method was used to detect the minimum inhibitory concentrations (MICs) of clinical conventional antibiotics, including ampicillin, ciprofloxacin, levofloxacin, cefepime, ceftazidime, ertapenem, imipenem, gentamycin, nitrofurantoin and tobramycin, and the results were interpreted in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) 2019 [25]. Furthermore, antimicrobial susceptibility testing of triclosan was performed by agar dilution method and according to previously study [19], isolates with an MICs value of > MIC₉₀ (Minimum drug concentrations at which 90% of strains were inhibited; MIC₉₀ = 0.5 µg/ml) were classified as triclosan-resistant. E. coli
ATCC 25922 served as the quality control strain for susceptibility testing.

**Serial passage experiments**

In order to determine whether there was cross-resistance between triclosan and other antibiotics in *E. coli*, serial passage experiments were conducted in vitro for triclosan-susceptible isolates DC8361, DC8363, DC8400, DC8413 and DC8510 as previously described [26]. Specifically, the isolates were cultivated on Maconkey ager plate and cultured overnight at 37 °C to obtain a single isogenic strain, which was then inoculated into 3ml fresh LB broth with different concentrations of triclosan at 37 °C overnight, the triclosan gradient concentrations were 0.0625, 0.125, 0.25, 1, 2, 4, 8, 16 and 32 µg/ml. Culture supernatants with bacteria growing in the highest triclosan concentrations were aspirated and continuously passaged in new triclosan gradients, after only four days of triclosan exposure, triclosan-mutant strains with MICs of not less than 0.5 µg/ml were obtained.

**Mutant Stability testing**

The stability of triclosan resistance was confirmed by continuous passage in vitro for triclosan-mutant strains. Briefly, the triclosan-mutant strains were cultured in 3 ml fresh LB broth without triclosan at 37 °C for 24 hours. Every 24 hours, 30 µl of overnight culture supernatants were transferred to another 5 ml tube containing 2.97 ml fresh LB broth without triclosan. After six cycles, the MICs of triclosan as well as ten antibiotics were tested in triplicate respectively using the same method described previously.

**Efflux pump inhibition testing**

To test efflux pump activity of triclosan-resistant *E. coli*, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was trialed. The resistant strains were tested on agar plates with the presence or absence of efflux pump inhibitor CCCP by the agar dilution method as described [25]. Test concentrations of CCCP were ensured with consideration to
determine the optimal subminimal inhibitory concentrations (sub-MICs) that could inhibit the overexpression of efflux pump without affecting the growth of bacteria. Compared with triclosan alone, MICs value of triclosan decreased by four times or more was confirmed having an inhibitory effect when triclosan was used in combination with the efflux pump inhibitors (CCCP, 10 μg/ml) [27].

**Polymerase chain reaction (PCR) detection of mutations in fabI gene**

Genome DNA of triclosan-resistant *E. coli* strains as well as randomly selected equal numbers of triclosan-susceptible strains, were extracted using the Biospin Bacterial Genomic DNA Extraction kit (Bioflux, Tokyo, Japan) according to the manufacturer's instructions. Then, *fabI* gene and 14 known drug efflux pump encoding genes (*ydcT, ydcU, ydcV, ydcS, cysP, cysU, marA, soxS, yhiv, acrB, acrD, acrF, mdfA and norE*) were amplified by PCR with the specific oligonucleotide primers, positive PCR products were directly sequenced by Shanghai Genomics Institute Technology Co. Ltd [15]. Genetic mutations were further analyzed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi; GenBank accession number: NC000913.3). Primer of *fabI* gene was listed in supplementary materials, Tables 3.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Analysis of the transcriptional levels was undertook using qRT-PCR on six different ABC transporters system encoding genes *ydcT, ydcU, ydcV, ydcS, cysP* and *cysU*; two Arac-regulator genes *marA* and *soxS*; four RND efflux pump encoding genes of the *yhiv* and *acrBDF*; the *fabI* gene which encoding for the enacyl carrier protein reductase during the bacterial fatty acid; the MdfA efflux Tolc, *mdfA*; and the NorE efflux pump encoding gene of the *norE*. Briefly, triclosan-resistant strains with overexpressing of the efflux pump activity were included, ATCC 25922 served as the control strain. The strains above were inoculated in fresh Luria broth (LB) and allowed to grow to logarithmic phase (OD$_{600}$ =
The total cellular RNA of these cultures was extracted using the Bacterial RNA Miniprep Kit (Biomiga, Shanghai, China) according to the manufacturer's recommendation. Subsequently, the purified RNA was subjected to reversely transcribed into cDNA by means of the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, MA, USA), amplification was performed using TB Green Premix Ex Taq II (Tli RNaseH Plus) (2×) (Takara, Japan). In the PCR reaction, a global gene \( \text{gapA} \) and housekeeping gene \( 16S \text{ rRNA} \) were used as corresponding internal control to calculate for quantification of transcriptional expression level of the efflux pump genes by the \( 2^{-\Delta\Delta Ct} \) method. According to Fatma Ibrahim Sonbol et al., the increase of 2-fold or greater indicated overexpression compared to the control strain ATCC 25922 [21]. Specific qRT-PCR primers were listed in supplementary materials, Tables 3. All experiments were performed in triplicates and the datas were displayed as the mean ± SD values (listed in supplementary materials, Tables 2).

**Genotyping by Multilocus sequence typing (MLST)**

All the triclosan non-susceptible isolates were typed by using MLST method. In short, the sequences of eight housekeeping gene (\( \text{trpB, uidA, dinB, icdA, pabB, polB, put and trpA} \)) of \( \text{E. coli} \) were amplified with specific primers available at the MLST database (https://bigsdb.pasteur.fr/index.html), and sequence types (STs) were evaluated by comparing the allelic profiles to the MLST database.

**Strain typing by pulse field gel electrophoresis (PFGE)**

To confirm and analyze the clonal relatedness of the triclosan-resistant isolates, PFGE was also used for analysis the clonal relatedness of the triclosan-resistant isolates according to the PulseNet protocols published by the US Centers for Disease Control and Prevention (CDC) with minor modifications. The cell suspensions treated with protease K were incubated with XbaI restriction enzyme at least for 2 hours at 37 ºC to digest the DNA
fragments. Then PFGE was performed using a CHEF-MAPPER XA PFG system (Bio-Rad, USA) for 18 hours. The detailed running condition were as follows: initial switch time value of 2.16 sec, final switch time of 54.17 sec at a gradient of 6 V/cm at a 120° included angle [28]. Next, the electrophoretic banding patterns were visualized by GelDoc XR gel imaging system (Bio-Rad, USA) and further analyzed by Quantity One (Bio-Rad Laboratories, USA). The Unweighted Pair Group Method with Arithmatic Mean (UPGMA) with optimization set at 1.5% to create the dendrogram, cut off line at 85% was considered to analyze genetic relatedness [29]. *Salmonella* standard strain H9812 was taken as the positive control.

**Abbreviations**

ATCC: American Type Cultures Collection; CLSI: Clinical and Laboratory Standards Institute; CCCP: Carbonyl cyanide 3-chlorophenylhydrazone; *E. coli*: *Escherichia coli*; MICs: Minimal Inhibitory Concentrations; MDR: Multidrug-resistant; MIC$_{90}$: Minimum drug concentrations at which 90% of strains were inhibited; MLST: Genotyping by Multilocus sequence typing; LB: Luria Broth; PCR: Polymerase chain reaction; PFGE: Strain typing by pulse field gel electrophoresis; qRT-PCR: Quantitative real-time polymerase chain reaction; sub-MICs: subminimal inhibitory concentrations; SSIs: surgical site infections; STs: Multilocus sequence types.

**Declarations**

**Ethics approval and consent to participate**

The need for ethics approval and consent is deemed unnecessary in this research according to the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

**Consent for publication**

Not applicable.
Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests

Funding

This work was financially supported by Planned Science and Technology Project of Wenzhou (no. Y20180193). The funder had no role in the design of the study and collection, analysis, and interpretation of data and writing of the manuscript.

Author's Contributions

WLZ carried out experiments, analyzed the data and wrote the manuscript. WYX, YX and WLL participated in experiments. YJZ and XKZ performed the results analysis and directed the drawing. CQX participated in analysis of results. JMC and TLZ designed the study and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Acknowledgements

Not applicable.

References

1. Riley LW. Pandemic lineages of extraintestinal pathogenic *Escherichia coli*. Clin Microbiol Infect. 2014;20(5):380-90.

2. Raeispour M, Ranjbar R. Antibiotic resistance, virulence factors and genotyping of Uropathogenic *Escherichia coli* strains. Antimicrob Resist Infect Control. 2018;7:118-25.

3. Alanazi MQ, Alqahtani FY, Aleanizy FS. An evaluation of *E. coli* in urinary tract infection in emergency department at KAMC in Riyadh, Saudi Arabia: retrospective study. Ann Clin Microbiol Antimicrob. 2018;17(1):1-7.
4. Sanchez GV, Babiker A, Master RN, Luu T, Mathur A, Bordon J. Antibiotic Resistance among Urinary Isolates from Female Outpatients in the United States in 2003 and 2012. Antimicrobial Agents and Chemotherapy. 2016;60(5):2680-3.

5. Walker E, Lyman A, Gupta K, Mahoney MV, Snyder GM, Hirsch EB. Clinical Management of an Increasing Threat: Outpatient Urinary Tract Infections Due to Multidrug-Resistant Uropathogens. Clin Infect Dis. 2016;63(7):960-5.

6. Yueh MF, Tukey RH. Triclosan: A Widespread Environmental Toxicant with Many Biological Effects. Annu Rev Pharmacol Toxicol. 2016;56:251-72.

7. Brett K AC. Triclosan in Single Use Medical Devices for Preventing Infections_ A Review of Clinical Effectiveness, Safety and Guidelines. Canadian Agency for Drugs and Technologies in Health. 2019 1-42.

8. Geens T, Neels H, Covaci A. Distribution of bisphenol-A, triclosan and n-nonylephenol in human adipose tissue, liver and brain. Chemosphere. 2012;87(7):796-802.

9. Pycke BF, Geer LA, Dalloul M, Abulafia O, Jenck AM, Halden RU. Human fetal exposure to triclosan and triclocarban in an urban population from Brooklyn, New York. Environ Sci Technol. 2014;48(15):8831-8.

10. Yin J, Wei L, Shi Y, Zhang J, Wu Q, Shao B. Chinese population exposure to triclosan and triclocarban as measured via human urine and nails. Environ Geochem Health. 2016;38(5):1125-35.

11. Ribado JV, Ley C, Haggerty TD, Tkachenko E, Bhatt AS, Parsonnet J. Household triclosan and triclocarban effects on the infant and maternal microbiome. EMBO Molecular Medicine. 2017;9(12):1732-41.

12. Xue J, Wu Q, Sakthivel S, Pavithran PV, Vasukutty JR, Kannan K. Urinary levels of endocrine-disrupting chemicals, including bisphenols, bisphenol A diglycidyl ethers, benzophenones, parabens, and triclosan in obese and non-obese Indian children.
13. Henly EL DJ, Maingay JB, Lacey MM, Smith TJ, Forbes S. Biocide Exposure Induces Changes in Susceptibility, Pathogenicity, and Biofilm Formation in Uropathogenic Escherichia coli. Antimicrob Agents Chemother. 2019:e01892–18.

14. Sun Y, Hu X, Guo D, Shi C, Zhang C, Peng X, et al. Disinfectant Resistance Profiles and Biofilm Formation Capacity of Escherichia coli Isolated from Retail Chicken. Microb Drug Resist. 2019;25(5):703-11.

15. Curiao T, Marchi E, Viti C, Oggioni MR, Baquero F, Martinez JL, et al. Polymorphic variation in susceptibility and metabolism of triclosan-resistant mutants of Escherichia coli and Klebsiella pneumoniae clinical strains obtained after exposure to biocides and antibiotics. Antimicrob Agents Chemother. 2015;59(6):3413-23.

16. Piddock LJ. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev. 2006;19(2):382-402.

17. Khan R, Zeb A, Roy N, Thapa Magar R, Kim HJ, Lee KW, et al. Biochemical and Structural Basis of Triclosan Resistance in a Novel Enoyl-Acyl Carrier Protein Reductase. Antimicrob Agents Chemother. 2018;62(8):e00648–18.

18. Lenahan M, Sheridan A, Morris D, Duffy G, Fanning S, Burgess CM. Transcriptomic analysis of triclosan-susceptible and -tolerant Escherichia coli O157:H19 in response to triclosan exposure. Microb Drug Resist. 2014;20(2):91-103.

19. Morrissey I, Oggioni MR, Knight D, Curiao T, Coque T, Kalkanci A, et al. Evaluation of epidemiological cut-off values indicates that biocide resistant subpopulations are uncommon in natural isolates of clinically-relevant microorganisms. PLoS One. 2014;9(1):e86669.

20. Kampf G. Biocidal Agents Used for Disinfection Can Enhance Antibiotic Resistance in Gram-Negative Species. Antibiotics (Basel). 2018;7(4):110-24.
21. Sonbol FI, El-Banna TE, Abd El-Aziz AA, El-Ekhawy E. Impact of triclosan adaptation on membrane properties, efflux and antimicrobial resistance of *Escherichia coli* clinical isolates. J Appl Microbiol. 2019;126(3):730-9.

22. Nicolae Dopcea G, Dopcea I, Nanu A, Diguta C, Matei F. Resistance and cross-resistance in *Staphylococcus* sp. after prolonged exposure to different antiseptics. J Glob Antimicrob Resist. 2019;1:19.

23. Li M, He Y, Sun J, Li J, Bai J, Zhang C. Chronic Exposure to an Environmentally Relevant Triclosan Concentration Induces Persistent Triclosan Resistance but Reversible Antibiotic Tolerance in *Escherichia coli*. Environ Sci Technol. 2019;53(6):3277-86.

24. Carey DE, McNamara PJ. The impact of triclosan on the spread of antibiotic resistance in the environment. Front Microbiol. 2014;5:e00780.

25. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing*. CLSI supplement M100 Wayne, PA: Clinical and Laboratory Standards Institute. 2019.

26. Lu J, Jin M, Nguyen SH, Mao L, Li J, Coin LJM, et al. Non-antibiotic antimicrobial triclosan induces multiple antibiotic resistance through genetic mutation. Environ Int. 2018;118:257-65.

27. Park YK, Ko KS. Effect of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on killing *Acinetobacter baumannii* by colistin. J Microbiol. 2015;53(1):53-9.

28. M RE, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, et al. Standardization of Pulsed-Field Gel Electrophoresis Protocols for the Subtyping of *Escherichia coli* O157:H7, Salmonella, and Shigella for PulseNet. FOODBORNE PATHOGENS AND DISEASE. 2006 3:59-67.

29. Cao X, Zhang Z, Shen H, Ning M, Chen J, Wei H, et al. Genotypic characteristics of multidrug-resistant *Escherichia coli* isolates associated with urinary tract infections.
Table 1. Mutations of fabI gene and MICs of triclosan and other antibiotic against *E. coli* strains

| Isolates | Triclosan MICs (μg/ml) | AMP  | CIP  | LVX  | FEP  | CAZ  | ETP  | IPM  | GEN  | NIT  | TOB  |
|----------|-----------------------|------|------|------|------|------|------|------|------|------|------|
| DC8358   | 8                     | >128 | 0.5  | 0.5  | 32   | 16   | 16   | 2    | >64  | 64   | >64  |
| DC8419   | 4                     | >128 | >32  | 64   | 16   | 16   | <0.5 | <0.25| >64  | 32   |      |
| DC8424   | 4                     | >128 | >32  | 16   | >64  | >64  | >32  | >32  | >64  | 64   | >64  |
| DC8603   | 2                     | >128 | >32  | >64  | 64   | 32   | <0.5 | <0.25| <2   | >128 |      |
| DC8724   | 8                     | >128 | >32  | 32   | 32   | 64   | 16   | 1    | >64  | 16   |      |
| DC8361   | 0.125                 | <4   | <0.25| 0.5  | <2   | <2   | <0.5 | <0.25| <2   | 16   |      |
| DC8363   | 0.125                 | >128 | <0.25| 0.5  | 2    | <2   | <0.5 | <0.25| <2   | <8   |      |
| DC8400   | 0.125                 | >128 | <0.25| <0.25| <1   | <2   | <0.5 | <0.25| <2   | 16   |      |
| DC8413   | 0.25                  | >128 | <0.25| <0.25| <1   | <2   | <0.5 | <0.25| <2   | <8   |      |
| DC8510   | 0.125                 | >128 | <0.25| <0.25| 2    | <2   | <0.5 | <0.25| <2   | <8   |      |

**a** MICs, Minimum inhibitory concentration.

AMP, ampicillin; CIP, ciprofloxacin; LVX, levofloxacin; FEP, cefepime; CAZ, ceftazidime; ETP, ertapenem; IPM, imipenem; GEN, gentamycin; NIT, nitrofurantoin; TOB, tobramycin.

**b** Gly, Glicine; Ala, Alanine; Thr, Threonine; Met, Methionine; Arg, Arginine; Val, Valine; Phe, Phenylalanine; Ser, Serine; Leu, Leucine; Asp, aspartic acid; Glu, glutamic acid.

**c** Bolded values point means resistance.

**d** ND, Not detected.

Table 2. Efflux pump phenotype testing
| Isolates | MICs (μg/ml) | Triclosan + CCCP (10 μg/ml) | fold changes |
|----------|--------------|----------------------------|--------------|
| DC8358   | 8            | 1                          | 8            |
| DC8419   | 4            | 1                          | 4            |
| DC8424   | 4            | 1                          | 4            |
| DC8603   | 2            | 2                          | 1            |
| DC8724   | 8            | 0.5                        | 16           |

* Compared with triclosan alone, MICs value of triclosan decreased by four times or more was confirmed having a positive efflux pump phenotype when triclosan was used in combination with the efflux pump inhibitors (CCCP, 10 μg/ml). + means strains with positive efflux pump phenotype. - means strains with negative efflux pump phenotype.

**Figures**
Relative expression level of fabI. Values of three biological repeats represent the mean ± SD. * means overexpression, that was the relative expression levels increased by 2-fold or greater compared to the control strain E. coli ATCC 25922. Compared to ATCC 25922, the expression of fabI was increased (> 2-fold) in DC8358 (fold-changes: 5.69 ± 0.49), DC8419 (fold-changes: 25.14 ± 0.42), DC8424 (fold-changes: 5.11 ± 0.43), DC8603 (fold-changes: 34.05 ± 0.23) and DC8724 (fold-changes: 41.85 ± 0.59).
Figure 2

Relative expression levels of genes. (A) The relative expression levels of efflux pump encoding genes in DC8358, the expression of ydcV (fold-changes: ydcV, 5.71 ± 0.68) was increased; (B) The relative expression levels of efflux pump encoding genes in DC8419, the expression of ydcV, yihV and acrB (fold-changes: ydcV, 8.74 ± 0.61; yihV, 3.57 ± 0.52; acrB, 3.44 ± 0.21, respectively) was increased; (C) The relative expression levels of efflux pump encoding genes in DC8424, the expression of ydcU, ydcS, yihV, acrD, and mdfA (fold-changes: ydcU, 4.71 ± 0.13; ydcS, 2.8 ± 0.42; yihV, 6.82 ± 0.65; acrD, 2.63 ± 0.14; mdfA, 5.13 ± 0.26, respectively) was increased; (D) The relative expression levels of efflux pump encoding genes in DC8724, the expression of ydcT, ydcU, ydcS, cysP, and yihV (fold-changes: ydcT, 6.56 ± 0.56; ydcU, 18.25 ± 1.36; ydcS, 8.76 ± 0.49; cysP, 3.89 ± 0.2; yihV, 2.00 ± 0.03, respectively) was increased. Values of three
biological repeats represent the mean ± SD. * means overexpression, that was
the relative expression levels increased by 2-fold or greater compared to the
control strain ATCC 25922.

Figure 3

PFGE and MLST profile of five triclosan-resistant E. coli isolates. Relatedness of
PFGE results was analyzed using QualityOne software (Bio-Rad Laboratories,
USA), and the phylogenetic tree was generated using UPGMA clustering, cut off
line at 85% was considered to analyze genetic relatedness. The result showed
differences in PFGE patterns and STs typing.

Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to
download.
Supplementary material.doc