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Triclosan Resistant Bacteria from Sewage Water: Culture Based Diversity Assessments and Co-Resistance Profiling to Other Antibiotics

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Triclosan (TCS) is an antimicrobial agent used in various human personal care products against both gram-positive and gram-negative bacteria. The purpose of this study was to evaluate the presence of TCS-resistant bacteria in sewage water in Peshawar, Khyber Pakhtunkhwa (KPK), Pakistan, for the first time. TCS-supplemented Luria Bertani (LB) agar was used to isolate TCS-tolerant bacteria. A total of 17 TCS-resistant isolates were randomly selected from a large pool of bacteria that showed growth on TCS-supplemented LB agar. Based on gram staining and physiochemical characteristics, the isolated strains were identified as *Salmonella* typhi (n = 6), *Escherichia coli* (n = 4), *Citrobacter freundii* (n = 4), *Proteus mirabilis* (n = 1), *Enterobacter cloacae* (n = 1), and *Pseudomonas aeruginosa* (n = 1). The Triclosan mean minimum inhibitory concentrations (MICs) for the isolates of *Salmonella* typhi, *Escherichia coli*, *Citrobacter freundii*, *Proteus mirabilis*, *Enterobacter cloacae*, and *Pseudomonas aeruginosa* were 23.66 µg ml^{-1}, 18.75 µg ml^{-1}, 42 µg ml^{-1}, 32 µg ml^{-1}, 64 µg ml^{-1}, and 128 µg ml^{-1}, respectively. The antibiogram revealed that all isolates were resistant to penicillin G (100%) and linezolid (100%), followed by ampicillin (94%), tetracycline (76%), tazobactam (76%), sulbactam/cefoperazone (64%), polymyxin PB (58%), amikacin (29.41%), aztreonam (29.41%), imipenem (5%), and gentamicin (5%). This is the first known study regarding the isolation of TCS-tolerant bacteria from sewage water in Peshawar, KPK, Pakistan. It was concluded that all the TCS-resistant isolates were multidrug resistant (MDR) gram-negative rod-shaped bacteria, mostly belonging to the *Enterobacteriaceae* family.

Keywords: **Triclosan, waste water, resistance, minimum inhibitory concentration, cross-resistance, antibiotics**

Introduction

Triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol] is a biocide used in numerous human care products, that inhibits the growth of broad range of microorganisms by targeting an enzyme called Enoyl Acyl Carrier (ENR) [1]. Four different ENRs such as FabI, FabK, FabL and FabV have been discovered till date but only FabI is known to be a potential target of Triclosan [2]. Numerous human personal care products such as soaps, toothpaste, detergents and shampoo contains varying concentration of TCS, usually 300 g/l which is 0.3% w/v, which prevent the growth of wide range of microorganisms such as bacteria, fungi and virus [3].

In developed countries, the overuse of TCS in personal care product, most importantly toothpaste and soaps, has resulted in the contamination of environment and

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can also be easily detected in the urine and plasma of large number of people [4]. A study conducted in the United States (US) has reported the level of TCS in urine of large number of adults, to be higher than the Minimum Inhibitory Concentration (MIC), stopping the growth of most bacteria [4]. TCS has recently been recognized as environmental endocrine disruptor and thus have negative effects on human health [5]. TCS can effectively inhibit tumor necrosis factor-α-stimulated urokinase production in human gingival fibroblasts [6]. TCS also possess potential cytotoxic, estrogenic and anti-estrogenic activities [7]. Several researchers have reported that triclosan can induce inflammatory responses in epithelial cells [8]. Furthermore, triclosan has also affected the aquatic ecosystem and the toxicity of triclosan towards fish, crustaceans, and algae have been demonstrated [9]. Certain plants are also able to accumulate TCS in their tissues as a result of irrigation with wastewater [10].

After use, the TCS containing common products reach wastewater treatment plants (WWTPs) via drainage system [11]. As wastewater treatment plants are not much efficient and TCS is a highly stable compound, a large amount of TCS is therefore released to receiving water bodies such as rivers, streams and canals etc. [12]. Wastewater treatment plants in US emit around 1.1×10^5 to 4.2×10^5 kg TCS to receiving water bodies [13]. A geological survey conducted in the US revealed that, in US streams, TCS was the most common detected compound [14].

It has been suggested that the continuous discharge of the TCS to environment can result in TCS tolerant bacteria by applying selective pressure on bacterial strain [15]. Bacteria are able to employ various mechanisms to cope with TCS such as activation of efflux pumps, mutation in the gene that codes for the target enzyme, increasing target expression and enzymatic inactivation and biodegradation of the biocide [16]. In addition, TCS has also been suspected to induce co-resistance or cross-resistance to other antibiotics [1]. It was suggested that TCS resistance in bacterial strains were linked to low level resistance to different antibiotics such as β-lactams, aminoglycosides, fluoroquinolones, ampicillins, chloramphenicol and tetracycline [17]. After exposing *Salmonella enterica* to TCS, reduction in susceptibility to other antibiotics was observed [18]. However, numerous studies have shown that there is no such link between TCS exposure and antibiotic resistance [15].

As TCS is known to affect microbial communities that are found in the natural environment hence, TCS tolerant bacteria are widely distributed in nature [19, 20]. TCS resistant coliform bacteria such as *Citrobacter freundii, Morganella morganii, Serratia fonticola* and *Serratia liquefaciens* were isolated from sewage sludge [21]. TCS tolerant potentially pathogenic bacteria were also reported from sewage and river water in the North-West, Potchefstroom, South Africa [22]. However, study regarding the evaluation of the presence of TCS tolerant bacteria in the natural environment of Pakistan has not yet been conducted.

Therefore, the aim of the current study was to isolate TCS resistant bacteria from sewage waste waters in Peshawar. Isolated TCS resistance strains were identified and characterized through various biochemical tests. In addition, minimum inhibitory concentration of TCS and Co-antibiotic susceptibility profile of the selected isolates were also determined. To the best of our knowledge this is the first report on the isolation of TCS resistant bacteria from natural environment in Pakistan.

Materials and Methods

Samples collection

Sewage water samples were serially diluted to a level of 10^{-9}. About 0.1 ml from each dilution tube was spread on nutrient agar (Oxoid Ltd., UK) plates. The plates were incubated at 30℃ for 24 h. After 24 h incubation, around 30 colonies were randomly picked based on morphological characteristics, from all the plates, and were two times sub-cultured on nutrient agar plates, to achieve pure cultures. Colonies having the same shape, appearance and size were picked once only.

Screening for triclosan resistance bacteria

Selected bacterial colonies were streaked on Luria Bertani Agar (LB) (Oxoid Ltd.) supplemented with various concentrations (0.1–10 μg/ml) of triclosan (Sigma-Aldrich, USA). These plates were incubated at 30℃ for 24 h. Colonies showing growth on TCS supplemented LB media were selected for further analysis.

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Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined by using the two-fold broth dilution method. A stock solution of TCS of 12.5 mg dissolved in 50 ml Dimethyl sulfoxide (DMSO), in doubling dilution range from 0–256 ug/ml TCS in Mueller Hinton broth (MHB) (Oxoid), made for each test. Turbidity of overnight bacterial culture was adjusted with 0.5 McFarland solution. A total of 0.3 ml of the adjusted inoculums was added to each tube containing 1 ml of antimicrobial agent in the dilution series. A tube containing broth was taken as positive control. The experiment was repeated on two different occasions and the mean values were reported. The MIC was determined as the lowest concentration that only inhibited visible bacterial growth after 24 h of incubation at 30℃.

Morphological and biochemical identification of selected isolates

The identification of triclosan resistance bacterial isolates were confirmed through morphological and biochemical characteristics such as Gram staining, Oxidase, Indole, Citrate, Voges Proskaeur (VP), Methyl Red (MR), Triple sugar iron (TSI) and Catalase test according to bergey's manual of determinative bacteriology [23].

Antibiotics susceptibility profile of triclosan resistance isolates

The antibiotic susceptibility profiles of all triclosan resistant isolates were determined by using Kirby Bauer Disc Diffusion method [24]. All the isolates were evaluated for resistance to eleven antibiotics: These belong to various classes of antibiotics, such as Tetracycline (30 μg), Polymyxin B (300 μg), Tazobactam (110 μg), Linezolid (30 μg), Ampicillin (25 μg), Gentamicin (10 μg), Imepenem (10 μg), Penicillin G (10 μg), Amikacin (30 μg), Aztreonam (30 μg), and Sulbactam/cefoperazon (105 μg). Fresh overnight broth culture of all the individual isolate were spread on Mueller-Hinton agar (Oxoid) and antibiotic disks were immediately placed on each plate. All the plates were incubated at 30℃ for 24 h. The zones of inhibition were measured and interpreted according to Performance Standards for Antimicrobial Disc Susceptibility Tests [25].

Results

Screening and identification of triclosan resistant bacteria

Among the 30 isolated strains, only 17 (56.66%) bacterial isolates were able to grow on TCS supplemented media. TCS resistant bacteria identified on morphological and biochemical characteristics are given in Table S1. The bacterial isolates were identified as *Salmonella* typhi (S2B, S2C, S2D, S3A, S3B, and S1E), *E. coli* (S1A, S3C, S2E, and S2F), *Citrobacter freundii* (S1C, S1D, S3E, and S3F), *Proteus mirabilis* (S2A), *Enterobacter cloacae* (S1B) and *Pseudomonas aeruginosa* (S3D). Amongst these *S.* typhi was the most predominant bacteria with prevalence of 35.30% followed by *E. coli* (23.52%), *Citrobacter freundii* (23.52%), *Proteus mirabilis* (5.9%), *Enterobacter cloacae* (5.9%) and *Pseudomonas aeruginosa* (5.9%).

Minimum inhibitory concentrations

The MIC for *E. coli* DH5α, which is a TCS tolerant strain and was used as a standard, was 0.5 μg/ml, and therefore bacterial isolates having MIC above 0.5 μg/ml were considered resistant, otherwise TCS tolerant. Interestingly, it was observed that all the isolates had MIC value of 1 or above. The mean MIC values are given in Table 1. The highest MIC was observed for *P. aeruginosa* (S3D) and *C. freundii* (S1D) (128 μg ml/l) followed by *E. coli* (S2E), *E. cloacae* (S1B) and *S.* typhi (S2B, S1E) (64 μg ml/l), *P. mirabilis* (S2A) and *C. freundii* (S1C) (32 μg ml/l), *E. coli* (S1A) and *S.* typhi (S3B) (8 μg ml/l), *S.* typhi (S3A) and *C. freundii* (S3E, S3F) (4 μg ml/l), *E. coli* (S2F) (2 μg ml/l), *E. coli* (S3C) and *S.* typhi (S2C, S2D) (1 μg ml/l) respectively.

Antibiotic susceptibility profiles

All the isolates showed multi drug resistance pattern (MDR) (Table 2). All of the *S.* typhi isolates (S2B, S2C, S2D, S3A, S3B and S1E) showed resistance to Ampicillin, Penicillin G, Tazobactam, Linezolid and Polymyxin B. These isolates were found completely susceptible to Amikacin, Gentamicin, Imipenem and Aztreonam. In addition, four isolates (S2B, S2C, S2D and S1E) were resistant to Tetracycline and three isolates (S2B, S2C and S2D) were resistance to Sulbactum/Cefaperazone. All the isolates of *E. coli* (S1A, S2E, S2F and S3C) were resistant to Penicillin G, Linezolid, Ampicillin, Tetra-

Table 1. Triclosan MIC distribution (µg /ml) of different bacterial species isolated from waste water of Peshawar.

S. No	Isolate	Bacteria	MIC (µg/ml)
1	Control	*E.coli* DH5α	0.5
2	S3D	*Pseudomonas aeruginosa*	128
3	S1D	*Citrobacter freundii*	128
4	S2E	*Escherichia coli*	64
5	S1B	*Enterobacter cloacae*	64
6	S2B	*Salmonella* typhi	64
7	S1E	*Salmonella* typhi	64
8	S2A	*Proteus mirabilis*	32
9	S1C	*Citrobacter freundii*	32
10	S1A	*Escherichia coli*	8
11	S3B	*Salmonella* typhi	8
12	S3A	*Salmonella* typhi	4
13	S3E	*Citrobacter freundii*	4
14	S3F	*Citrobacter freundii*	4
15	S2F	*Escherichia coli*	2
16	S3C	*Escherichia coli*	1
17	S2C	*Salmonella* typhi	1
18	S2D	*Salmonella* typhi	1

cycline, Tazobactam and Sulbactum/Cefaperazone and were completely sensitive to Gentamicin and Imipenem. Only 2 isolates (S2E and S2F) showed resistance to Amikacin and 3 isolates (S1A, S2E and S3C) were found resistant to Polymyxin B and Aztreonam each. All the isolates of *C. freundii* (S1C, S1D, S3E, and S3F) showed resistance to Ampicillin, Penicillin G, Tetracycline, and Linezolid and were sensitive to the remaining tested antibiotics. Single *P. aeruginosa* isolate (S3D) was found resistant to Penicillin G, Tazobactam, Tetracycline, Ampicillin and Linezolid and was found susceptible to the remaining tested antibiotics. The only isolate of *E. cloacae* (S1B) exhibited resistance to all classes of tested antibiotics except Sulbactam/cefoperazon, Polymyxin B and Amikacin. The *Proteus mirabilus* isolate (S2A) isolate resisted to all the tested antibiotics except Imipenem, Gentamicin and Aztreonam.

Discussion

The use of TCS in various human personal care products poses a high risk to public safety and environment as large amount of TCS and other biocide are discharged to different water bodies. The present study attempted to isolate TCS resistant bacteria from two heavily polluted water canals *i.e.,* Board Bazar canal and Umar Gul road canal, present in highly populated areas in Peshawar, Khyber Pakhtunkhwa, Pakistan. TCS resistant isolates from all the 2 different regions were gram negative rod shape bacteria. Except a single isolate of the *Pseudomonas* spp., all the isolates belong

Table 2. Antibiotic susceptibility profile of triclosan resistant isolates.

Antibiotics	*Salmonella* typhi species (6 isolates)		*Escherichia coli* Species (4 isolates)		*Citrobacter* species (4 isolates)		*Pseudomonas aeruginosa* species (1 isolate)		*Enterobacter cloacae* specie (1 isolate)		*Proteus mirabilis* species (1 isolate)	
	%R	%S	%R	%S	%R	%S	%R	%S	%R	%S	%R	%S
Penicillin G	100	0	100	0	100	0	100	0	100	0	100	0
Linezolid	100	0	100	0	100	0	100	0	100	0	100	0
Ampicillin	100	0	100	0	100	0	100	0	100	0	100	0
Tetracycline	66.66	33.34	100	0	100	0	100	0	100	0	100	0
Tazobactam	100	0	100	0	0	100	100	0	100	0	100	0
Sulbactam/cefoperazon	50	50	100	0	0	100	0	100	0	100	100	0
Polymyxin	100	0	75	25	0	100	0	100	0	100	100	0
Amikacin	0	100	50	50	0	100	0	100	0	100	100	0
Aztreonam	0	100	75	25	0	100	0	100	100	0	0	100
Imipenem	0	100	0	100	0	100	0	100	100	0	0	100
Gentamicin	0	100	0	100	0	100	0	100	100	0	0	100

a) %R: Percentage of Resistant isolates, b) %S: Percentage of susceptible isolates.

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to various genera of *Enterobacteriaceae* family. These results were not unexpected since gram-negative bacteria in general are more resistant to TCS because of the ability of their outer membrane to act as barrier, thus allowing many gram-negative bacteria to develop intrinsic resistance to various disinfectants and antiseptics [26]. In addition, it has been noted that the rate of antimicrobial resistance in *Enterobacteriaceae* family is higher than Gram-positive bacteria [27].

All the TCS tolerant bacterial spp, showed elevated level of MICs. *P. aeruginosa* (S3D) and *C. freundii* (S1D) were the most resistant spp, among all the isolates. The presence of FabV isoenzyme in *P. aeruginosa* has been shown responsible for this increase in MICs [13]. The precise determination of a specific concentration of TCS is therefore, very important as different microbes respond differently to a particular concentration of TCS [15]. The different degree of MICs shown by different bacterial strains is attributed to various factors such as duration of exposure to TCS, concentration of TCS and bacterial strain [28]. Bacteria are able to employ efflux pumps when an unwanted substance enters bacterial cell and more efflux pumps can be activated [15]. Bacteria can develop different resistance mechanisms when they are exposed to antimicrobials [15]. It has been suggested that TCS can act as substrate for multi-drug efflux pumps [29]. Various studies have demonstrated that variety of efflux play role in TCS resistance in different bacterial strains [22]. Gram negative rods like *E. coli, P. aeruginosa* and *S. enterica* serovar Typhimurium have been shown to employ resistance-nodulation-cell division (RND) pumps for the removal of TCS from inside of the cell [26]. In addition, members of the *Enterobacter* genera are known to use various efflux mechanism to remove TCS from the cell [26].

The current study also showed that all the TCS resistant isolates were multi drug resistant. Penicillin G and Linezolid were the most ineffective antibiotics as almost all the isolates were resistant to both of these antibiotics while Imipenem and Gentamicin were the most effective antibiotics tested. It has been suggested that exposure to TCS may also lead to development of cross-resistance to other antibiotics in bacteria [26]. It was demonstrated by J. Lu *et al*., 2018, that a wild type *E. coli* became multi drug resistance after continuous exposure to various concentrations (0, 0.02, 0.2 and 2 mg/l) of TCS for 30 days [30]. A similar study reported the induction of a multidrug resistant *A. baumannii* strain ZJ06m after continuous exposure to TCS [31]. However, Cottell A *et al.,* 2009, have shown that antimicrobial tolerance and antibiotic resistance have no linkage. In their study they determined the antibiotic susceptibility of TCS resistant strains of *E. coli*, *S. aureus* and *A. johnsonii* by comparing it with their sensitive counterpart strains and concluded that none of the TCS tolerant strains showed resistance to antibiotics nor they tended to have smaller zones of inhibition compared to the sensitive counterpart strains [32]. In addition, another similar study [4] reported, in case of *E. coli*, a zero correlation, between the minimum inhibitory concentration of triclosan and cross resistance between any of the tested antibiotics. Therefore, it is suggested to perform further studies to clearly establish link between TCS and antibiotic resistance.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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