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Isolation and characterization of antibiotic-resistant bacteria from pharmaceutical industrial wastewaters

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ABSTRACT

Contamination of surface waters in underdeveloped countries is a great concern. Treated and untreated wastewaters have been discharged into rivers and streams, leading to possible waterborne infection outbreaks which may represent a significant dissemination mechanism of antibiotic resistance genes among pathogenic bacterial populations.

The present study aims to determine the multi-drug resistance patterns among isolated and identified bacterial strains in a pharmaceutical wastewater effluent in north Tunisia. Fourteen isolates were obtained and seven of them were identified. These isolates belong to different genera namely, *Pseudomonas*, *Acinetobacter*, *Exiguobacterium*, *Delftia* and *Morganella*. Susceptibility patterns of these isolates were studied toward commonly used antibiotics in Tunisia. All the identified isolates were found to have 100% susceptibility against colistin sulfate and 100% resistance against amoxicillin. Among the 11 antibiotics tested, six patterns of multi-drug resistance were obtained. The potential of the examined wastewater effluent in spreading multi-drug resistance and the associated public health implications are discussed.

1. Introduction

Chronic exposure of bacteria to antibiotics can cause antibiotic resistance and associated hospital-acquired infections. In recent years, antibiotic contamination is recognized as an emerging environmental pollution in aquatic environments, because of their potential adverse effects on the ecosystem and human health [1,2]. The majority of antibiotics used to treat infection in humans, livestock animals and plants are excreted into the environment as their intact parent compounds via various pathways, including wastewater effluent discharge, runoff from land to which agricultural or human waste has been applied, and leaching [3].

During the last fifteen years, pharmaceuticals have been receiving great attention, as they are highly biologically active compounds. Among these residues, antibiotics have attracted a large share of attention [2], among these residues, antibiotics attracts more attention in many countries in the world. Since, veterinary and humans antibiotics residues are continuously introduced into the environment by manufacturing industries, Wastewater Treatment Plant (WWTP), which can have an impact on the water quality, the ecosystem and the human and animal health [4,5]. It has been reported in the literature that wastewater pose a real environment problems [6]. Antibiotic residues in wastewater effluents pose a serious environmental concern as they are recalcitrant against various wastewater treatment processes. Besides, numerous antibiotics are persistent in the environment and are not totally eliminated, which can represent a harmful effect on human and animal health [7,8]. The discharge of antibiotics in aquatic environments may adversely affect bacterial communities, leading to have several types of resistance to antibiotics. The resistance to antibiotics is determined genetically by antibiotic resistance genes, (ARGs), which are detected in various water environments [3]. It has been proven that many antibiotics can

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influence the occurrence of antibiotic resistance in water ecosystem environments [3,9–11].

The aim of the present work was to study the bacterial diversity in pharmaceutical effluent collected from Tunisian industry and to evaluate the antibiotic resistance patterns of bacteria which were isolated and identified.

2. Materials and methods

2.1. Wastewater samples collection

Samples of wastewaters were collected from a pharmaceutical industry located in northern Tunisia. This industry is specialized in the antibiotic fabrication. The samplings were carried out from the outgoing wastewater industry for 3 consecutive months: February (S1), March (S2) and April (S3). Samples were collected in amber glass bottles and immediately transported to the laboratory. After sampling and filtering through 1 μm membrane filters (Euro Scientific, Belgium), the samples were handled immediately or kept in the dark at 4 °C until analysis.

2.2. Bacteriological study

2.2.1. Enumeration of total cultivable and antibiotic-resistant bacteria in pharmaceutical wastewater

Bacteriological analyses were performed using the membrane filtration method which is described by Novo and Manaia [12]. In fact, a non-selective agar (nutrient agar) culture medium is recommended for the examination of total resistant bacteria. The respective antibiotic-resistant populations were enumerated on the media supplemented with 32 mg/L of amoxicillin (AML) or 4 mg/L of ciprofloxacin (CIP) which belong to the only two families (Beta-lactams and quinolones) produced by this industry. The antibiotic concentrations used were determined in previous studies, as adequate to recover antibiotic resistant bacteria [13]. Volumes of 1–100 ml (culture medium with antibiotic) and of 1–10 ml of water samples or decimal dilutions thereof (culture medium without antibiotic) were filtered through cellulose nitrate membranes (0.45 μm pore size, 47 mm diameter, Albet), which were placed onto the culture medium described above and incubated for 48 h at 30 °C. All analyses were made in triplicate. After the incubation period, the number of colony forming units (CFU) was registered on the basis of filtering membranes containing between 10 and 100 CFU.

2.2.2. Culture-independent approach: DGGE analysis

Total DNA was extracted from pharmaceutical wastewaters and the denaturing gradient gel electrophoresis (DGGE) was carried out on the 16S rDNA hypervariable V3–V5 regions. PCR was effectuated using the primers 907 R (5′-CCGTCAATTCCTTTGAGTTT-3′) and 357F (5′-CTACGGGAGGCAGCAG-3′). An additional 40 bp GC-rich sequence (GC-clamp) was added to the end of primer 357F in order to prevent complete strand separation and to stabilize fragment migration. The amplification program consisted of an initial denaturing step at 94 °C for 4 min, 10 cycles of 94 °C for 30 s, 61 °C for 1 min and 72 °C for 1 min, 20 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 1 min. PCR products were resolved on 6% (w/v) polyacrylamide gels in 1X TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA (pH 7.4)) using a denaturing gradient ranging from 40 to 60% (where 100% denaturant contained 7 M urea and 40% formamide) [14]. Gels were run at 100 V for 17 h at 60 °C. Agarose and polyacrylamide gels were stained, respectively, for 10 and 30 min in a 0.5 mg L^{-1} solution of ethidium bromide, washed with sterile distilled water and immediately photographed with a digital capture system (GelDoc Cleaver). The DGGE band profiles were analyzed with an image analyzing system Image J software (version 1.46) and XLSTAT that allows the conversion of the bands density and migration into numerical values called principal components.

2.2.3. Isolation and identification of antibiotic resistant bacteria

According to the method described by Sturm [15], bacteria were isolated from one sample (April sample) chosen at random using the mineral medium composed with (mg L^{-1}): KH_2PO_4, 85; K_2HPO_4, 208; $Na_2HPO_4.2H_2O$, 334; NH_4Cl, 5; $CaCl_2$, 27.5; $MgSO_4$, $7H_2O$, 22.5; $FeCl_3$, $6H_2O$, 0.25, the pH was adjusted to 7 supplemented with 1% of effluent and 1% of antibiotic solution (4 mg L^{-1} of ciprofloxacin and 32 mg L^{-1} of amoxicillin were added separately) as the sole carbon source. Flasks were incubated for 21 days at 30 °C on rotary shaker at 200 rpm.

The isolated bacteria were identified basing on the analysis of the 16S rRNA gene sequence and using the primers 27F and 1492R [16]. After a serial dilution in mineral medium, individual colonies were selected and purified by repeated streaking on culture medium containing; mineral medium and 1% of antibiotic solution. Genomic DNA from pure strains was extracted by sodium dodecyl sulfate (SDS)-proteinase K treatment [14]. Molecular amplification of the 16S–23S ITS region and the 16S rRNA gene were performed by using respectively the universal primers S-D-Bact-1494-a-20, L-D-Bact-0035-a-15 and 50-S-D-Bact-0008-a-S-20- 30 and 50-S-D-Bact-1495-a-S-20-30 [17]. The amplification reaction mixture consisted of 1 × PCR reaction buffer, 2.5 mM MgCl2, 0.12 mM deoxynucleoside triphosphate, 0.2 mM of each primer, 1 U Taq DNA polymerase and 1 ml of total DNA. The PCR program consisted of an initial step at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 45 s, annealing for 1 min at 55 °C and elongation for 2 min s at 72 °C, followed by final elongation step at 72 °C for 8 min. The ITS-PCR amplification patterns and 16S products were migrated respectively on standard 2% agarose gels in 0.5 × Tris–borate–EDTA buffer and stained for 30 min in 0.5 mg/L ethidium bromide solution. The amplified 16S rRNA fragments were sequenced and identified by comparison with those available at the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) using the BLAST program [18]. A phylogenetic dendrogram was constructed by the neighbor joining method and tree topology was evaluated by performing boot-strap analysis of 1000 data sets using MEGA 4.1 [19].

2.2.4. Determination of antibiotic resistance profiles

Antimicrobial susceptibility testing was conducted on Mueller–Hinton agar plates (Difco, Becton–Dickinson, Sparks, MD) by the agar disc-diffusion method according to CLSI criteria (2007) [20]. The following antimicrobial agents were tested (μg/disc): nalidixic acid (30), ciprofloxacin (5), amoxicillin (25); ticarcillin (75); cephalothin (30); ceftazidime (30); streptomycin (10); sulphamethoxazole/trimethoprim (25); tetracycline (30); gentamicin (10) and colistin sulfate (50). For the antibiotics Amoxicillin and colistin sulfate, which are not included in the CLSI list, the following criteria were used: $S \geq 21/R < 14$ and $S \geq 10/R < 10$, respectively. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as a control strains.

3. Results

3.1. Total cultivable and antibiotic-tolerant bacteria

Given the expected high abundance of antibiotic resistant bacteria in pharmaceutical wastewaters, culture media was supplemented with amoxicillin or ciprofloxacin in order to facilitate the isolation of beta-lactam- and quinolone-resistant bacteria. The