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ORIGINAL PAPER

UPLC-MS/MS analysis of antibiotics in pharmaceutical effluent in Tunisia: ecotoxicological impact and multi-resistant bacteria dissemination

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Abstract

The UPLC MS/MS analysis showed the presence of the two antibiotics in the pharmaceutical industry discharges during 3 months; norfloxacin and spiramycin which were quantified with the mean concentrations of 226.7 and 84.2 ng mL^{-1}, respectively. Sixteen resistant isolates were obtained from the pharmaceutical effluent and identified by sequencing. These isolates belong to different genera, namely *Citrobacter, Acinetobacter, Pseudomonas, Delftia, Shewanella*, and *Rheinheimera*. The antibiotic resistance phenotypes of these isolates were determined (27 tested antibiotics-discs). All the studied isolates were found resistant to amoxicillin and gentamicin, and 83.33% of isolates were resistant to ciprofloxacin. Multiple antibiotic resistances were revealed against β-lactams, quinolones, and aminoglycosides families. Our overall results suggest that the obtained bacterial isolates may constitute potential candidates for bioremediation and can be useful for biotechnological applications. Genotoxic effects were assessed by a battery of biotests; the pharmaceutical wastewater was genotoxic according to the bacterial Vitotox test and micronuclei test. Genotoxicity was also evaluated by the comet test; the tail DNA damages reached 38 and 22% for concentrated sample (10×) and non-concentrated sample (1×), respectively. However, the histological sections of kidney and liver's mice treated by pharmaceutical effluent showed normal histology and no visible structural effects or alterations as cytolysis, edema, or ulcerative necrosis were observed. Residual antibiotics can reach water environment through wastewater and provoke dissemination of the antibiotics resistance and induce genotoxic effects.

Keywords Pharmaceutical effluent · Antibiotic multi-resistance · Genotoxicity · UPLC-MSMS · Biotests.

Introduction

Antibiotic residues can reach the environment through wastewater discharges (Heberer 2002; Yan et al. 2013). Wastewater treatment plants, pharmaceutical companies, and aquaculture activities are the sources of the aquatic antibiotic contamination (Adelowo et al. 2008; Baquero et al. 2008; Drewnowski and Makinia 2014; Hockenreiner et al. 2015). Antibiotics used in human medicine and animal use are not completely metabolized (Rang et al. 2003) and these active products can easily reach the sewage treatment systems. Since limitations were noted for the complete elimination

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of antibiotics residues in the process of conventional wastewater treatment, special attention was accorded to the evaluation of the environmental antibiotics risks (Czekalski et al. 2012; Novo et al. 2013; Varela et al. 2014).

Antibiotic residues at trace levels may be harmful due to their intrinsic biological activity, even after chronic exposure (Hernando et al. 2006). Perturbations in the productivity, disturbances of the ecosystem and biodiversity, and food chain disruptions may take place as a result of the abnormal physiological and biochemical functions of some major primary and secondary producers touched by antibiotics contamination (Halling-Sorensen 2000; Wollenberger et al. 2000). Another major issue is that the exposure to a contaminated effluent highly loaded with antibiotics helps to spread the resistance genes in environmental bacterial communities (Chen et al. 2013; De Castro et al. 2014; Kristiansson et al. 2011). Induction of resistance is possible even at low concentrations (Gullberg et al. 2011). The massive and uncontrolled use of antibiotics can lead to the spreading of incurable diseases caused by resistant bacteria (Solomons 1978) and aggravate the situation of immune-compromised persons (Allen et al. 2010; Canton 2009; De Costa et al. 2011).

To address such problems, genotoxicity tests using bacteria, plant, and animal cells have been developed (Ma et al. 2012; Mater et al. 2014; Zegura et al. 2009).

The combination of analytical chemistry and biological methods is advisable to predict the complex toxicological effects of compounds in the environment. The aim of this work is to determine the levels of the antibiotics residues in pharmaceutical effluent samples by LC MSMS, to study the resistant bacteria community in the effluent, and to assess cytotoxic and genotoxic effects of pharmaceutical effluent by a battery of biotests.

Materials and methods

Pharmaceutical effluent sampling

Pharmaceutical effluent samples were collected from a pharmaceutical industry located in Zaghouen (Tunisia). During 3 months (February, March, and April 2013), wastewaters were sampled before the discharging point using an instantaneous sampling. The samples were collected in autoclaved glass bottles and immediately transported to the laboratory and kept in the dark at 4 °C until microbiological, chemical, and toxicological testing.

Analytical methodology

UPLC-MS/MS analysis was performed to determine 56 antimicrobial drugs in the pharmaceutical effluents. After spiking with appropriate internal standards, a 100 mL aliquot was concentrated on an Oasis® HLB cartridge. After elution, drying, and reconstitution, an 8ùL aliquot was injected for analysis. UPLC-MS/MS analysis was performed using a Waters Acquity UPLC® system interfaced with a Xevo TQ-S tandem quadrupole mass spectrometer, all from Waters (Milford, MA, USA). Compounds were separated on an Acquity UPLC® BEHC18 column (100 × 2.1 mm) using 0.1% formic acid in water and in acetonitrile as mobile phase. Chromatography was performed within 12 min using a gradient elution.

Microbial study

Culture-independent approach: DGGE analysis

Total DNA was extracted from pharmaceutical wastewater and stored at − 20 °C. The denaturing gradient gel electrophoresis (DGGE) was performed on the 16S rDNA hypervariable V3–V5 regions. The primers 907 R (5′-CCGTCA ATTCCTTTGAGTTT-3′) and 357F (5′-CTACGGGAG GCAGCAG-3′) were used for PCR. The primer 357F was enriched with an additional 40 bp GC-rich sequence (GC-clamp sequence) to avoid the complete strand separation. The PCR program included 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) (Ettoumi et al. 2010). Gels were run at 100 V for 17 h at 60 °C. An ethidium bromide solution (0.5 mg L^{-1}) was used for staining the agarose and polyacrylamide, respectively, for 10 and 30 min. The gels were washed with sterile distilled water and immediately photographed with a digital capture system (GelDoc Cleaver).

Culture-dependent approach

Enumeration of antibiotic-resistant bacteria in the pharmaceutical effluent Enumeration of antibiotic-resistant bacteria was carried out using the membrane filtration method which is described by Novo and Manaia (2010). The respective antibiotic-resistant populations were enumerated on the media supplemented with 32 mg L^{-1} of amoxicillin (AMX) or 4 mg L^{-1} of ciprofloxacin (CIP); β-lactams and quinolones are the only two antibiotics families produced by this industry.

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 2 days of incubation, the number of colony forming units (CFU) was determined on the basis of filtering membranes containing between 10 and 100 CFU.

Isolation and selection of antibiotic-resistant bacteria According to the method described by Sturm (1973), bacteria were isolated from one wastewater sample (similar chemical composition of wastewaters) 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 3 weeks 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 (Lane 1991). 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 (Ettoumi et al. 2010). Molecular amplification of the 16S-23S ITS region and the 16S rRNA gene were performed 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. The TS sequences are highly conserved in the areas encompassing the rRNA genes and relatively stable regions located at the ends of the spacer (Daffonchio et al. 2000). 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^{-1} 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 (Altschul et al. 1990). A phylogenetic dendrogram was constructed by the neighbor joining method and a tree topology was evaluated by performing boot-strap analysis of 1000 data sets using MEGA 4.1 (Kumar et al. 2004).

Determination of antibiotic resistance profiles Antibiotic resistance patterns were performed on Mueller–Hinton agar plates using the agar disc-diffusion method according to CLSI criteria (2007) (CLSI 2007). Twenty-seven antimicrobial agents were tested (µg/disc): nalidixic acid (30), ciprofloxacin (5), amoxicillin (25); ticarcillin (75); cephalothin (30); ceftazidime (30); streptomycin (10); sulphamethoxazole/trimethoprim (1.25/23.75); tetracycline (30); gentamicin (15); colistin sulfate (50); tobramycin (10); amoxicillin/clavulanic acid (20/10); piperacillin (75); cefotaxime (30); penicillin (6); oxacillin (5); erythromycin (15); spiramycin (100); cefoaxime (30); imipenem (10); ertapenem (10); levofloxacin (5); tigecycline (15); amikacin (30); ticarcillin/clavulanic acid (75/10); ofloxacin (5). 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 25,922 and *Pseudomonas aeruginosa* ATCC 27,853 were used as a control strains.

Toxicological study

Cell culture

Human hepato-cellular liver carcinoma cells (C3a) were grown in Dulbecco's modified Eagle's culture medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (1% penicillin–streptomycin). The cultures were incubated in humidified atmosphere conditions with 5% CO_2 at 37 °C. Every 4 days, the cultures were trypsinized and divided into 100 000 cells per Petri dish of 35 mm diameter with 10 mL of culture medium. A bath of Milli-Q water was also used to preserve humidity.

Concentration of samples

A 100 mL of water samples was concentrated on the previously preconditioned Oasis® HLB cartridge. After the drying process, the analytes were eluted and evaporated to dryness. Then, the concentrated residues were mixed with Milli-Q water so as to obtain the four dilutions and they were analyzed with the comet assay and the micronuclei test. Thus, the tested concentrations were 1×, 2.5×, 5× and 10×.

Vitotox test

The test is performed with Salmonella Typhimurium TA104 that is genetically modified and it shows DNA damage by emitting a readily detectable signal of bioluminescence after exposure to mutagenic factors (Verschaeve et al. 1999). This

is made possible by the insertion of a *luciferase* gene (the *lux* operon of *Vibrio fischeri)* under transcriptional control of a mutated *recN* gene (TA104 *recN2-4* or *Genox* strain). A second control strain (TA104 *pr1* or *Cytox* strain) was also used. In this case, *lux* expression was independent of DNA damage and the light production increase was not the direct result of DNA damage, whereas the decreased light production reflected toxicity. Overnight bacteria cultures were conducted for the both *Genox* and *Cytox* strains in rich medium containing 625 µL of tetracycline (0.8 mg mL^{-1}) and 312.5 µL of ampicillin (8 mg mL^{-1}). Cultures were placed in a water bath of 36 °C for 16 h at 300 rpm. After the overnight incubation, the cultures were placed on ice for 15 min. 20 mL of mineral medium and 160 µL of previously cultured bacteria were added and stirred during 1 h in an appropriate water bath (300 rpm, 36 °C). The *Genox* and *Cytox* work cultures are ready for testing; a mixture of 2.125 mL of mineral medium, 350 µL of bacterial suspension, and 1 mL of S9 was prepared and transferred to a black 96-well microplate with 100 µL from each sample. When the S9 is not used in the test, we added 1 mL of mineral medium. Positive controls were used; 4-NQO (0.4 mg mL^{-1}) tested without S9 and B(a)P (0.8 mg mL^{-1}) tested with S9.

A microplate luminometer was used to measure genotoxicity and toxicity and the light was analyzed every 5 min over a 4-h time span. The signal-to-noise ratio (S/N) represents the calculation of the exposed bacteria light production divided by non-exposed bacteria light production for each measurement and for each strain separately. All the calculations were done automatically and they were based on measurements taken between 60 and 240 min of incubation. The previous experiments have demonstrated that genotoxicity always take place when the maximum S/N (*genox*)/max S/N(*cytox*) is greater than 1.5 and cytotoxicity is assumed when S/N in *Genox*/*Cytox* decreases far below 0.8 (Verschaeve et al. 1999, 2012; Verschaeve 2002).

Comet assay

The genotoxicity of a tested substance was valued by the determination of DNA damages in the comet tail. This test was conducted according to standard procedures (Fairburn et al. 1995; Tice et al. 2000). Four dilutions of wastewaters were tested on C3a cells. For this, 4×10^5 cells were seeded into 24-well plates and incubated at 37 °C (5% CO_2) overnight. The culture medium was also removed, and the wastewater samples was then added and incubated again overnight at the same conditions. Ethyl methanesulfonate (EMS) (0.75 mM) was deployed as the positive control, whereas unexposed cells controls were also included as negative control.

In the next step, 75 µL of a cell-LMP suspension (0.8% low melting point agarose) was laid on the slides that had been previously frosted and then coated with 1% NMP (normal melting point agarose) and eventually they were left to dry. A lysis buffer (2.5 M NaCl; 100 mM EDTA; 10 mM TRIS) was used for cellular lysis. Alkali unwinding of the DNA was realized through the slides incubation in electrophoresis buffer (0.3M NaOH, 1 mM EDTA) for 40 min in a horizontal electrophoresis unit, and then, a neutralization with a Tris buffer (0.4 M, pH 7.5) and an electrophoresis (20 min, 1 V/m, 300 mA) were conducted. So as to stain DNA, a DAPI (fluorescent dye) was used, and finally, the analysis of the slides was carried out with an Axio Imager.Z2 (Zeiss) fluorescence microscope with Metacyte and Metafer 4 (version 3.8.5) software from Metasystems (Altlussheim, Germany) to quantify DNA damage. Two gels were prepared for each concentration along with the analysis of 50 cells per gel. Furthermore, the DNA percentage in the comet tail was considered to measure the DNA damage. Comet assay data (tail intensity) of wastewater exposed and control cells were compared with the Mann–Whitney *U* test.

Micronucleus test

The micronucleus assay determines the DNA damaging capacity of substances by the micronucleus formation after treatment of cells with the potentially mutagenic agent in culture. The micronucleus rate, which represents either chromosome fragments or a whole chromosome, requires cell division to be formed and is taken as an indicator for the DNA damaging capacity of the tested substance (Fenech and Crott 2002).

The human line C3a cells were trypsinized, seeded in 24-well plates (2×10^5 cell/well), and incubated overnight at 37 °C (5% CO_2). The cells were exposed to four dilutions (previously described) of pharmaceutical effluent and cultivated again overnight at 37 °C (5% CO_2). Methyl methanesulfonate (MMS) was used as a positive control and unexposed cells as the negative control. After an overnight exposure, the medium was withdrawn and replaced by a medium containing cytochalasin B (4.5 µg mL^{-1}). Cells were fixed with a fresh cold solution of methanol and acetic acid (3/1) (v/v). The tubes containing the cells were kept well closed at − 20 °C for at least one night before spreading on slides. A DAPI DNA-staining was used to visualize under Zeiss light microscope and the acridine orange to visualize the cell shape. Per dilution, 2×10^3 C3a cells were scored. The cytochalasin B proliferation index (CBPI) was calculated as: CBPI = (1x mononuclear cells + 2x binucleated cells + 3x polynucleated cells)/total number of cells (Kirsch-Volders et al. 2003). Kastenbaum and Bowman tables were used for the analysis of the micronucleus results (Kastenbaum and Bowman 1970).

Histological study

The BALB/C mice used in the experiments were female about 10 weeks (weight ≈ 30 g) obtained from the Pasteur Institute in Tunis. The mice were grouped in ten per cage. They were synchronized for 15 days in a clean room with an alternating cycle: 12 h of light and 12 h of dark. The room temperature was maintained at 22 °C and a relative humidity of 50%. Mice were treated by intraperitoneal injection with a dose of 10 mL/kg to test the pharmaceutical effluent. The doses tested are previously determined sublethal. The ultrapure water sterilized by autoclaving was used as negative control.

Group 1: contains ten mice treated by intraperitoneal injection of a dose of 10 mL kg^{-1} of wastewater collected from pharmaceutical industry.

Group 2: contains ten mice treated by intraperitoneal injection of a dose of 10 mL kg^{-1} of ultrapure water sterilized by autoclaving. This group constitutes the negative control of the experiment.

Livers and kidneys were collected from mice for histological study. The sections were stained with haematoxylin–eosin.

Results

UPLC-MS/MS analysis

Two antibiotics were detected and quantified in the water samples, i.e., norfloxacin at 226.7 ng mL^{-1} and spiramycin at 84.2 ng mL^{-1}, and the presence of norfloxacin and spiramycin was noticed in the four effluent samples at different levels.

Microbiological study

Culture-independent approach

DGGE culture-independent technique was used to determine the bacterial community in pharmaceutical effluent sampled during 3 consecutive months. A noticeable diversity was observed by the analysis of the hypervariable region profiles of the 16S rRNA (V3–V5) on polyacrylamide denaturing gel (Fig. 1). The profiles can be divided into three parts, non-rich bands in GC part characterized with short migration (I), moderately rich in GC part characterized by medium migration (II), and in the third part, the bands are rich in GC and the migration is long (III). Most bands of amplified 16S rRNA were found in part III; this showed the high GC content in bacteria. We observed a large variability in DGGE bands intensities and in migration distances. A remarkable difference was noted between the three profiles

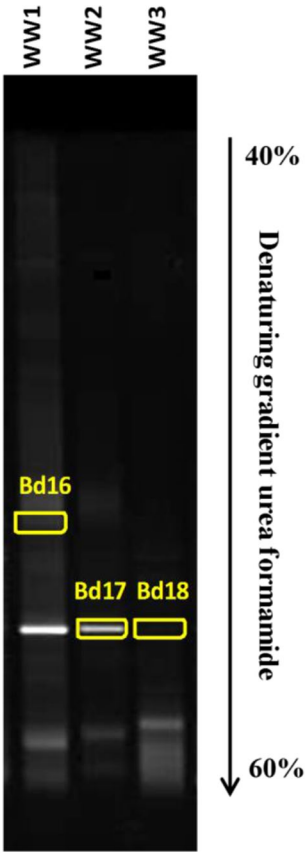

Fig. 1 DGGE profiles of three pharmaceutical effluent samples showing the variation of the bacterial population based on variable region V3–V5 of 16S rDNA

representing the months of February, March, and April. Three bands (Bd1, Bd2, and Bd3) were chosen and identified by sequencing from the three wastewater DGGE profiles. The two DGGE Bd1 and Bd3 bands were identified as uncultured bacteria and the DGGE Bd3 was identified as *Rhodocyclaceae* bacterium (Table 1).

Isolation and selection of antibiotic-degrading bacteria

The cultivable-resistant bacteria were determined from pharmaceutical effluent in culture media supplemented separately with amoxicillin (AMX, 32 mg L^{-1}) or ciprofloxacin (CIP, 4 mg L^{-1}). The resistant bacteria community to ciprofloxacin was reduced compared to resistant bacteria community to amoxicillin, 2.33×10^2 CFU mL^{-1} and 5.33×10^2 CFU mL^{-1}, respectively (Table 2).

Sixteen strains were isolated from pharmaceutical effluent collected in April on mineral medium 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. These antibiotic-resistant strains were identified by amplification of the ribosomal

Table 1 Identification of DGGE bands of bacteria in the sampled pharmaceutical effluent

Bands	Accession number	Sequence similarity (%)	Affiliation	Length (bp)	Origin	Sampling period
Bd16	KX815457	100	Uncultured bacterium	460	Wastewater	February
Bd17	KX815445	100	*Rhodocyclaceae* bacterium	458	Wastewater	March
Bd18	KX815446	99	Uncultured bacterium	348	Wastewater	April

Table 2 Enumeration of antibiotic-resistant bacteria in the pharmaceutical effluent

Tests	Medium culture + AMX	Medium culture + CIP
1	5	3
2	6	2
3	5	2
Total	5.33	2.33

intergenic transcribed spacer between the 16S and the 23S rRNA genes (ITS-PCR). The ITS profiles showed reproducible patterns consisting of 1 to 4 bands ranging from 150 to 950 bp. Six different ITS haplotypes were produced (data not shown).The most represented pattern was haplotype H3, found in 4 isolates, and composed by two reproducible bands of 950 and 750 bp. The other ITS groups were found in two isolates. Six representative strains are selected from the different groups for the partial 16S rRNA gene sequencing. On the basis of BLAST analysis of 16S rRNA gene similarity, six different genera including *Pseudomonas, Acinetobacter, Citrobacter, Shewanella* (the most represented in our collection (43%)), *Delftia*, and *Rheinheimera* were identified (Table 3).

The susceptibility of the six haplotypes to 27 antimicrobial agents is shown in Table 4. A high level of resistance was detected to amoxicillin (100%), gentamicin (100%), and ciprofloxacin (83.33%). A medium level of resistance was observed to cephalothin (66.66%), ceftazidime, tobramycin and cefotaxime (33.33%), and ticarcillin

Table 3 Identification of antibiotic-resistant bacteria isolated from pharmaceutical effluent

Strains	Accession number	Sequence similarity (%)	Length (bp)	Affiliation	Isolates number
Acinetobacter calcoaceticus	KP691594	100	437	Gamma-proteobacteria	1
Rheinheimera tangshanensis	KP691576	100	427	Gamma-proteobacteria	2
Citrobacter freundii	KP691572	99	499	Gamma-proteobacteria	4
Pseudomonas putida	KP691569	99	463	Gamma-proteobacteria	1
Delftia lacustris	KP691592	100	479	Beta-proteobacteria	1
Shewanella putrefaciens	KP691583	100	516	Gamma-proteobacteria	7

Table 4 Antibiotic resistance patterns observed for bacterial isolates obtained from pharmaceutical effluent

Strains	Resistance patterns	No. of antibiotics resistance	MAR
Acinetobacter calcoaceticus	CIP–TIC–AMX–CEF–CAZ–GMI	6	3/6
Delftia lacustris	AMX–GMI–TMN–AKN–CIP	5	3/6
Rheinheimera tangshanensis	PEN–AMX–OXA–FOX–ERY–SPN–GMI–OFX	8	3/6
Citrobacter freundii	AMX–AMC–TIC–PIP–CEF–FOX–GMI–TMN–CIP	9	3/6
Pseudomonas putida	CIP–AMX–CEF–GMI	4	3/6
Shewanella putrefaciens	CIP–TIC–AMX–CEF–CAZ–GMI	6	3/6

NA Nalidixic acid, *CIP* ciprofloxacin, *AMX* amoxicillin, *TIC* ticarcillin, *CEF* cephalothin, *ACZ* ceftazidime, *STR* streptomycin, *SXT* sulphamethoxazole/trimethoprim, *TET* tetracycline, *GMI* gentamicin, *CLT* colistin sulfate, *TMN* tobramycin, *AMC* amoxicillin/clavulanic acid, *PIP* piperacillin, *FOX* cefotaxime, *PEN* penicillin, *OXA* oxacillin, *ERY* erythromycin, *SPN* spiramycin, *CFX* cefotaxime, *IMP* imipenem, *ETP* ertapenem, *LVX* levofloxacin, *TGC* tigecycline, *AKN* amikacin, *TCC* ticarcillin/clavulanic acid, *OFX* ofloxacin, *MAR* multiple antibiotics resistance, *MAR* no. of resistance classes/no. of classes tested

(50%). Lower levels were detected to spiramycin, penicillin, oxacillin, amikacin, erythromycin, amoxicillin/clavulanic acid, and piperacillin (16.66%). All the studied isolates have not shown susceptibility to colistin sulfate, nalidixic acid, streptomycin, sulphamethoxazole/trimethoprim, tetracycline, imipenem, ertapenem, levofloxacin, tigecycline, and ofloxacin. An antibiotic multiresistance phenotype was observed. Three dominated multi-resistance profiles were detected for all the identified strains against the families of β-lactams, quinolones, and aminoglycosides.

Toxicological study

Vitotox test

The results showed that the tested pharmaceutical effluent is screened genotoxic and the max *Genox*/*Cytox* ratio was greater than 4 (Table 5) and greater than the positive controls (4-NQO and B(a)P) values. Genotoxicity decreased with addition of S9 mix to reach 2.2. It should be noted that no reduction of bioluminescence was perceived in the *Cytox* strain, which indicated that the effluent is not cytotoxic.

Comet assay

The DNA damages in the comet tail were measured in 100 cells. The results for comet tail length showed significant differences (Mann–Whitney *U* test) between tested concentrations, negative control (unexposed cells), and positive control (EMS 0.5 mM) (Fig. 2). The pharmaceutical effluent samples showed a dose-dependent genotoxic effect with tail DNA contents reaching 38 and 22% for the concentrated sample (10X) and non-concentrated sample (1X). It can be seen that this test confirmed the Vitotox results found previously.

Table 5 Genotoxic effects of pharmaceutical effluent on Salmonella Typhimurium TA 104 determined by the Vitotox test

Samples	Maximum S/N (*genox*)/maximum S/N (*cytox*) ratio-S9	Maximum S/N (*genox*)/maximum S/N (*cytox*) ratio+S9
PE	4.0	2.2
4-NQO	3.1	–
B(a)P	–	2.0

Genotoxicity always take place when the maximum S/N (*genox*)/maximum S/N(*cytox*) is greater than 1.5 and cytotoxicity is assumed when S/N in *genox*/*cytox* decreases far below 0.8

PE pharmaceutical effluent, *NC* Milli-Q water, *positive samples* 4-NQO tested without S9 and B(a)P tested with S9

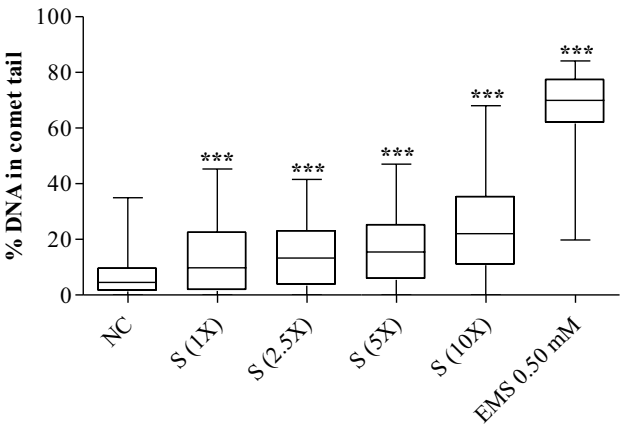

Fig. 2 DNA damage expressed as % DNA in comet tail from C3a cells that were exposed to different concentrations (1×, 2.5×, 5× and 10×) of pharmaceutical effluent

Micronuclei test

The micronucleus test results are showed in Table 6. It can be noted that the tested concentrations of pharmaceutical effluent were all genotoxic in a dose-dependent manner. Micronuclei in binuclear cells increased from 9.5 to 13 in non-concentrated sample and 10-× concentrated sample, respectively. The cytochalasin B proliferation index (CBPI) proportionally related to the detected number of micronuclei and reflects the degree of genotoxicity. For our pharmaceutical effluent, the CBPI rose from 1.2 to 1.59 for the non-concentrated sample and 10× concentrated sample, respectively.

Histological study

No significant difference was observed when compared histological sections of the mice's kidney and liver slides treated with sterile deionized water (as a negative control) and slides treated with pharmaceutical industry. In this present research, histological sections of mice's kidney and liver showed no visible structural effects or alterations as cytolysis, edema, fibrosis, or hemorrhagic necrosis (Fig. 3).

Discussion

The antibiotic consumption is increasing, and as a result, environmental residues are growing (Deschamps et al. 2012). Water is frequently contaminated by drug residues (Hussain et al. 2016). The detection and the quantification of antibiotics may be useful to understand the potential associated hazards. Many published studies have focused on the

Table 6 Micronuclei assay results of four different concentrations (1×, 2.5×, 5×, and 10×) of the pharmaceutical effluent

Samples	Tested concentrations of effluents	Binuclear cells with micronuclei (2000 cells)	Cytochalasin B proliferation index (CBPI) (500 cells)
PE	1×	9.5	1.20
	2.5×	12.5	1.25
	5×	14	1.50
	10×	13	1.59
NC	–	3	1.20
PC	–	16.5*	1.68

Statistics according to Kastenbaum and Bowman tables (Kastenbaum and Bowman 1970)

PE pharmaceutical effluent concentrations (1×, 2.5×, 5× and 10×), *NC* negative control (unexposed cells), *PC* positive control (MMS)

*Significative difference ($p < 0.01$). Results are given per 2000 analyzed cells

research of antibiotics in wastewaters (Dinh et al. 2011; Dorival-Garcia et al. 2013; Tahrani et al. 2016; Zhou et al. 2012).

The analysis for determination of residual levels of the 56 selected antibiotics by liquid chromatography mass spectrometry method operating in the positive ion mode ESI^+ showed the presence of norfloxacin and spiramycin in the studied effluent. The study of Hussain et al. (2016) reported that the residual levels of ofloxacin, ciprofloxacin, and levofloxacin were found in the range 0–9.40 mg L^{-1} in the pharmaceutical wastewaters in Lahore (Pakistan) compared to the highest concentration of norfloxacin which was determined in our research (0.29 mg L^{-1}) and the highest concentration was observed for ofloxacin from fluoroquinolones class as found in our study. This is consistent with reports by Collado et al. (2014) and Gros et al. (2010) who detected high levels of fluoroquinolones. The absence of other antibiotics could be due to the low solubility in the water. Their hydrophobic nature drives them to partition from water to particulate matter and sediments. A seasonal (Birosova et al. 2014; Li et al. 2011) and temporal (Musolff et al. 2009) differences in pharmaceutical wastewater concentrations were observed. Our research confirmed these differences and we have noted that in 3 months, the temperature increased between winter season and spring season, because there was no change in the production schedule of the pharmaceutical plant. The removal of these two antibiotics in the effluent by the industry was very low and these antibiotics reached the environment. The chemical properties/structures of antibiotics are the fundamental factor for efficient removal. In general, the removal efficiencies were observed for norfloxacin were approximately between 5 and 78% (Gulkowska et al. 2008). Macrolides antibiotics (spiramycin) demonstrated either mediocre removal or no elimination, and they are usually characterized by higher concentrations in effluent wastewaters. This study confirms results found by the previous researchers (Gobel et al. 2007; Gros et al. 2010). In some cases, an increase in antibiotic concentrations in the final effluent was noted and this may be explained by two hypotheses, the first, it is due to the deconjugation reactions during the treatment process (Miao et al. 2002), and the second, it is due to the modifications in the molecules adsorption behavior during the treatment processes (Lindberg et al. 2005).

It is well known that pharmaceutical industries contributed to the aquatic pollution by discharging contaminants. The studied industry is located in a non-urban area and releases their discharges directly into a ditch in contact with animals and plants. Moreover, the pharmaceutical wastewater discharges join the river Maliane around 1 km far of the industry. Dumping of untreated pharmaceutical wastewater constitutes an incontestable environmental threat (Rao et al. 2001). 95% of antibiotic molecules may be released unchanged into the wastewaters treatment plants and they can later reach the aquatic environment. Microbial community represents the first natural target of the antibiotic contamination (Goni-Urriza et al. 2000; Halling-Sorensen et al. 1999). As reported in the studies of Li et al. (2011) and Novo et al. (2013), bacteria community's dynamics depends on the geo-physical conditions such as temperature, pH, light, and flow (Hall et al. 2008; Sigee 2005; Zhang et al. 2011). The *Rhodocyclaceae* bacterium identified by DGGE technique in the studied effluent sampled in March is characterized by very versatile metabolic capabilities and degrades a wide range of carbon sources; this species lives in aquatic habitats and prefers oligotrophic conditions, occurs in waste water, and plays an important role in biological remediation at contaminated sites by polycyclic aromatic hydrocarbons (Oren 2014; Singleton et al. 2015). The DGGE showed the rearrangements of the aquatic bacterial populations in correlation with the variations of antibiotics levels in the investigated industrial pharmaceutical effluent.

In the previous studies of Lateef in 2003 and Tahrani et al. (2015), the resistant bacteria found per millimeter of pharmaceutical effluent were higher than those presented in this study. Some isolates (*Acinetobacter, Citrobacter*, and

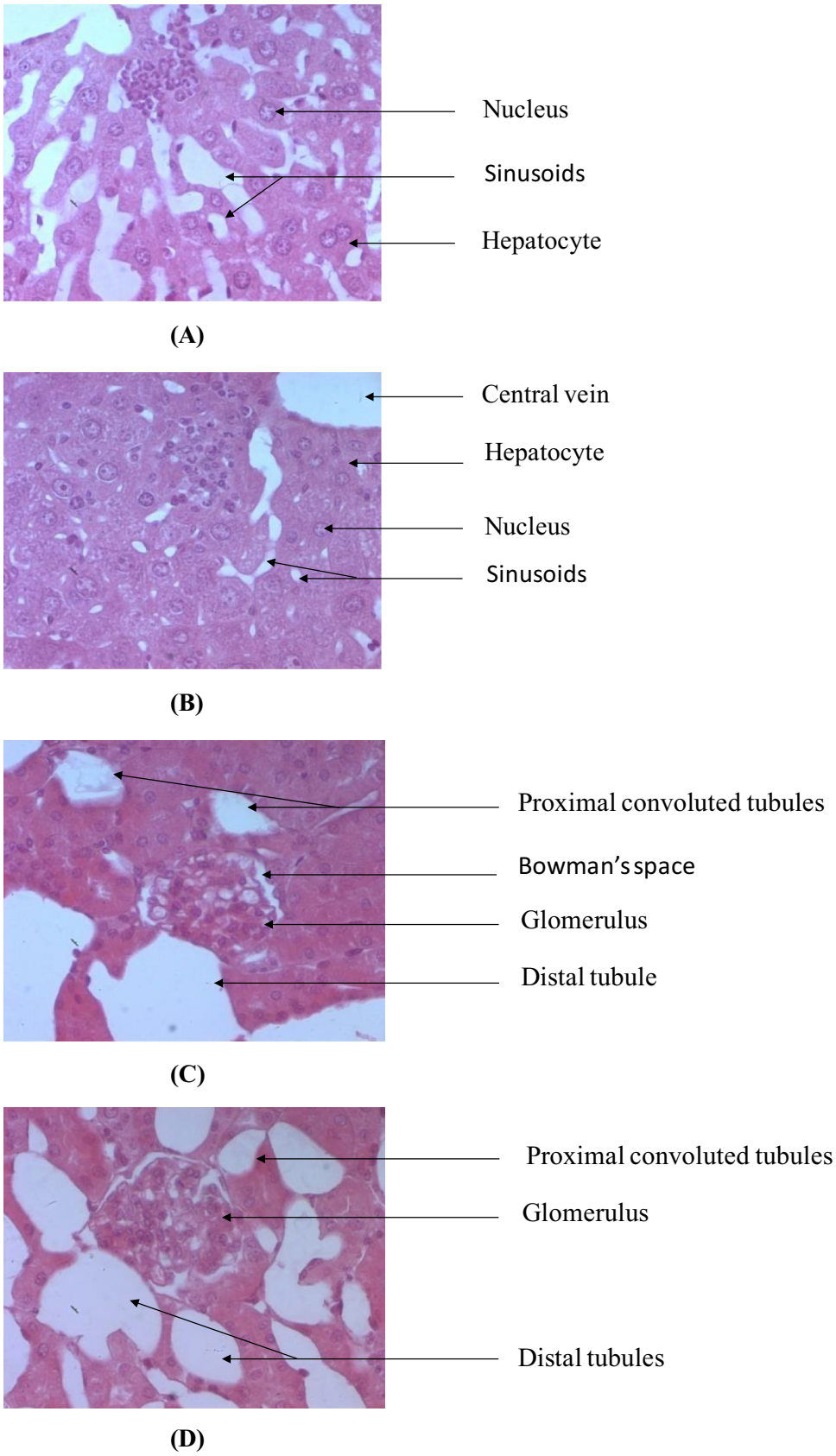

Fig. 3 Histological slides of mice's liver and kidney observed at high magnification (400×) with the light microscope

Shewanella) are potential pathogens capable of causing a variety of diseases in the respiratory and gastrointestinal tracts, infections in the urinary and hepatobiliary tracts, meningitis, bacteremia, otitis, soft-tissue infection (Albrecht et al. 2006; Ohara et al. 2000; Oh et al. 2008). In one hand, the isolation of pathogens from the pharmaceutical effluent is worrisome, but in the other hand, this may be an interesting result, because these pathogens are able to use the antibiotics as sole carbon source in the growth medium and subsequently a degradation of these micropollutants. Many previous studies have focused on the biodegradation of antibiotics with bacteria and optimization of the optimal conditions for this biodegradation (Dhall et al. 2012; Frank-Peterside et al. 2011; Ibegbulam-Njoku et al. 2013).

As reported in the previous studies, antibiotic contamination promoted the spreading of resistance and even multi-resistance of pathogens (Baquero et al. 2013; Gillings 2013). Human health can be also affected (Daughton and Ternes 1999; Martinez 2009; Tennstedt et al. 2005; Watkinson et al. 2007; Zhang et al. 2009).

Resistant bacteria in contaminant environmental water with antibiotics have been identified in several studies (Birosova et al. 2014; Figueira et al. 2011; Marti et al. 2014; Pei et al. 2006). Bacteria in these polluted aquatic matrices share or exchange transferable elements of DNA with other bacteria, this can occur between different bacterial species (Amos et al. 2014; Kaplan et al. 2013; Marti et al. 2014). The presence of antibiotic residues in the environment imposes selective forces for the emergence of antibiotic-resistant pathogens (Bengtsson-Palme and Larsson 2016). Eventually, some pathogens are related with confirmed or suspected nosocomial agents and infectious diseases. Furthermore, the resistant bacteria could find their way into drinking waters and food chain (Watkinson et al. 2007). It is a worldwide health threat which results in 700 000 deaths every year (Bengtsson-Palme and Larsson 2016). The ecological explanation of antibiotic resistance is the adaptation of the bacteria in the polluted environment (Finley et al. 2013; Pruden et al. 2013). Antibiotics such as fluoroquinolones provoke horizontal gene exchanges of resistance between bacterial community members, effects that could be detected even at low concentrations (Beaber et al. 2004; Larsson et al. 2007). Resistance is encoded by genes located in chromosomes and in extra-chromosomal materials. The cell division and the conjugation insure the passage of the resistance to the next generation between bacterial populations (Schluter et al. 2007; Wellington et al. 2013). In several studies, the multi-resistance phenomena were detected in aquatic environment containing synthetic antibiotics or modified molecules (Ash et al. 2002; Watkinson et al. 2007).

No alterations were noted by the histological study and the effects of the studied effluent were ultra-structural and non-visible or detectable in histological plan. However, the pharmaceutical effluent showed a genotoxic effect. Three toxicological tests: Vitotox, comet, and micronucleus assays were used and they were performed by microorganisms and eukaryote cells. The comet assay was often used, because this indicator test can be applied on wastewaters and it may reveal DNA damages induced by different mechanisms (Kudlak et al. 2016; Sharif et al. 2016). Finally, the micronucleus test is deployed, because it determines both structural and numerical chromosome aberrations and is currently considered one of the most significant and reliable genotoxicity tests (Kaur and Dua 2016; Kirsch-Volders et al. 2011; Parolini et al. 2016). The presence of antibiotics residues in the environment can pose serious health problems (Kungolos et al. 2006; Zegura et al. 2009). Hazardous effects were reported in several studies (Hernando et al. 2006; Sarmah et al. 2006). Maranho et al. (2014) demonstrated that pharmaceutical pollutants can alter aquatic organisms and distort the natural functions. Antibiotics are used to treat a number of human and animal infections (Verschaeve et al. 1999). Nevertheless, when they reach the aquatic environment they ended up being able to provoke genotoxicity in a genetically modified bacterial strain of Salmonella Typhimurium (Hartmann et al. 1998). Both eukaryotic tests (micronucleus and comet) confirm the results found by the prokaryotic test Vitotox in this research. The obtained results are interesting; insofar bioassays based on cells can provide a complete profile of the biological activities of chemical mixtures, as revealed in the study of Escher and Leusch (2012).

This research was not focused on the toxic activities of each molecule separately, but in the aquatic environment, molecules are present as mixtures of various therapeutic classes and the determination of toxic impact should take in consideration these pharmaceutical cocktails (Pomati et al. 2008). Numerous previous researches demonstrated that environmental cocktails elicited toxic alterations when an individual molecule proved not toxic (Cleuvers 2003, 2004). The toxic effect could be due to the formation of residues in the treatment processes. Indeed, many technologies used in municipal treatment such as disinfection by chlorination or UV irradiation, ultrafiltration, reverse osmosis, and ozone are able to induce genotoxic compounds (Bourgeous et al. 2001; Qin et al. 2005; Takanashi et al. 2009). As reviewed in the study of Mathur et al. (2007) that biological treatment can produce new mutagens.

Conclusion

The presence of the antimicrobial drugs in the pharmaceutical effluent, which reaches environment as an emerging environmental pollutant, leads to the spreading of the resistant pathogens and the alteration of the microbial diversity. Genotoxic effects on prokaryotes and eukaryotes were also

noted in this study. The treatment processes adopted by the studied pharmaceutical industry were not efficient enough, since residues of antibiotics were detected in the outgoing wastewater of the industry. The antibiotic-degrading isolates may be used for the bioremediation in contaminated aquatic environment.

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