Multiresistant opportunistic pathogenic bacteria isolated from polluted rivers and first detection of nontuberculous mycobacteria in the Algerian aquatic environment

Lydia Neïla Djouadi, Okba Selama, Ahmed Abderrahmani, Amel Bouanane-Darenfed, Lamia Abdellaziz, Meriam Amziane, Marie-Laure Fardeau and Farida Nateche

ABSTRACT

Opportunistic infections constitute a major challenge for modern medicine mainly because the involved bacteria are usually multiresistant to antibiotics. Most of these bacteria possess remarkable ability to adapt to various ecosystems, including those exposed to anthropogenic activities. This study isolated and identified 21 multiresistant opportunistic bacteria from two polluted rivers, located in Algiers. Cadmium, lead, and copper concentrations were determined for both water samples to evaluate heavy metal pollution. High prevalence of Enterobacteria and non-fermentative Gram-negative rods was found and a nontuberculous *Mycobacterium* (NTM) strain was isolated. To the best of our knowledge, this is the first detection of NTM in the Algerian environment. The strains were tested for their resistance against 34 antibiotics and 8 heavy metals. Multiple antibiotics and heavy metals resistance was observed in all isolates. The two most resistant strains, identified as *Acinetobacter* sp. and *Citrobacter freundii*, were submitted to plasmid curing to determine if resistance genes were plasmid or chromosome encoded. *Citrobacter freundii* strain P18 showed a high molecular weight plasmid which seems to code for resistance to zinc, lead, and tetracycline, at the same time. These findings strongly suggest that anthropized environments constitute a reservoir for multiresistant opportunistic bacteria and for circulating resistance genes.

Key words | circulating resistance genes, environment, multiresistance, nontuberculous mycobacteria, opportunistic bacteria

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INTRODUCTION

Opportunistic bacteria are normally nonvirulent bacteria that take advantage of an immune system depression to express their pathogenicity. A compromised immune system may result from recurrent infections, advanced human immunodeficiency virus (HIV) infection, genetic predisposition, and medical treatments such as immunosuppressive agents for organ transplant recipients, chemotherapy for cancer, or long-term antibiotic treatments (Fishman 2013). More generally, opportunistic bacteria can cause disease when the host's defense is weakened, whatever the reason and the duration of the host's failure. This is particularly true for hospital-acquired diseases, called nosocomial infections, which cause increasing mortality rates and healthcare costs, worldwide (Lynch *et al.* 2007).

In Algeria, 9,103 people were diagnosed HIV-positive in 2014 and the corresponding prevalence rate was estimated at 0.1%. The number of HIV infections is considered as relatively stable as 700 to 800 new cases have been diagnosed annually since 2009 (UNAIDS 2014). Nevertheless, diabetes is the most worrying immunodepressive condition in Algeria

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as 4.4 million people were living with this chronic disease in 2012. Furthermore, the prevalence of diabetes is continually increasing (Lamri *et al*. 2014). Consequently, the potential candidates of opportunistic infections are being constantly augmented in Algeria.

One of the most problematic characteristics of opportunistic pathogens is that they usually display low susceptibility to antibiotics, which makes them particularly hard to eradicate (Martinez 2009). Therefore, the impact of opportunistic infections has notably increased in the last two decades and many opportunistic bacteria are now being considered as emerging pathogens.

Epidemiological studies report that predominant opportunistic infection-associated bacteria include *Enterobacteriaceae* and non-fermentative Gram-negative rods, Gram-positive *Staphylococci*, *Enterococci* and *Micrococci*, and some genera of the *Actinobacteria* group (Cabrera *et al*. 2011). These past few years, nontuberculous mycobacteria (NTM) have emerged as a major cause of life-threatening infections in immunocompromised subjects, and have been recognized as emergent opportunistic pathogens since 2004 by the World Health Organization (Falkinham 1996; Giulieri *et al*. 2011).

A major part of these opportunistic bacteria are defined as ubiquitous since they possess a great metabolic versatility allowing them to adapt to various environments, such as polluted and non-polluted waters, soils and many other ecological niches (Berg *et al*. 2014).

Microbial aquatic ecosystems, mainly those exposed to anthropogenic activities, represent important vehicles for the dissemination of human-associated microorganisms and may constitute a reservoir for the spread of resistance genes among bacterial communities (Amos *et al*. 2014). Indeed, as a consequence of uncontrolled discharge of waste products from hospitals and industries, aquatic environments are principal recipients of contaminant residues such as antibiotics and heavy metals. The contaminants present in these waters constitute a selective force in bacterial communities which may acquire heavy metals and antibiotics resistance genes allowing them to survive. It has been suggested that genes encoding resistance to heavy metals can be located together with antibiotic resistance genes on either the same genetic structure (such as plasmids or integrons), or different genetic structures within the same bacterial strain. Moreover, resistance to heavy metals has been reported to enhance the antibiotic resistance ability of bacteria (Chen *et al*. 2015). Antibiotics overused in hospitals and heavy metals used in industry are, thus, creating a disturbance in microbial ecosystems to which bacteria adapt by developing abilities to resist and survive.

Oued El-Harrach, in east Algiers, and Oued Beni-Messous, in west Algiers, both receive effluents from many industries and hospitals and from domestic sewage. These two rivers discharge directly into the Mediterranean Sea, without any treatment. The aim of this study was to investigate the presence of multiresistant opportunistic bacteria in these two polluted rivers in Algiers, allowing us to estimate the bacteriological pollution threat to immunocompromised human subjects.

METHODS

Sampling sites

Oued El-Harrach is one of the largest rivers in the Algiers region. It originates in the Blidean Atlas (near Hamam Melouane) and discharges into the Mediterranean Sea, after a 67 km-long course. Oued El-Harrach is located in east central Algiers, one of the most heavily populated regions of Algeria, and is known to be a highly polluted river as it receives all the domestic effluents of the principal communes of the capital. Significant industrial activity takes place near this river (mainly in the Baba Ali and Central Algiers industrial zones), comprising paper, metallurgy and tannery industries as well as plastic and hydrocarbons transformations. All these industries are responsible for chemical pollution, especially with heavy metals. As well, Oued El-Harrach receives the Zemirli hospital effluent which may contain antibiotic residues.

Oued Beni-Messous is a smaller river located in the western suburb of Algiers. It extends from the Beni-Messous commune to 'Les Dunes' beach, where it discharges into the sea, and has a total length of about 10 km. Oued Beni-Messous is located in a densely populated and industrialized area. It receives the domestic sewage of all the neighboring communes (Beni-Messous, Cheraga, Dely-Ibrahim, and Bouzareah) and is directly polluted by the effluents of the

Beni-Messous hospital. The industrial activity near the river mainly consists of food (meat and milk transformation), metallurgical, and tannery industries which are known to generate heavy metal pollution. In the Oued Beni-Messous area pharmaceutical industries are also located and which probably cause the release of drug residues (as antibiotics or antiseptics).

The locations of the rivers are shown in Figure 1.

Sample collection

In order to avoid the dilution effect caused by rainfall, we chose to carry out the sampling campaigns during the dry period of the year. Accordingly, the collecting campaign took place in May, 2014. For each site, two samples were collected from two different points: one defined as the starting point (36°44′17,75″N; 03°07′46,26″E and 36°46′54,58″N; 02°54′46,28″E, for Oued El-Harrach and Oued Beni-Messous, respectively), shown in Figure 1 (points A and B), and the second from about 20 to 30 meters upstream from the starting point. The samplings were carried out from 60-cm depth in sterile 1-L glass bottles which were transported immediately to a laboratory where they were processed within 4 hours.

Physicochemical analysis and determination of heavy metal concentrations

Water temperature and pH were measured *in situ* using a portable pH meter (HI 98128, HANNA Instruments) whereas cadmium, copper, and lead concentrations were determined using a SOLAAR MQZ Zeeman air-acetylene flame atomic absorption spectrometer (Thermo Fisher Scientific).

Multiresistant opportunistic bacteria isolation

Opportunistic bacteria belong to a broad range of phylogenetic groups which possess diverse metabolic exigencies. Thereby, rich media which enable the growth of a great majority of bacteria, including exigent ones, have to be used. For this purpose, we chose to cultivate the samples on Brain Heart Infusion Agar (BHIA, Difco). In order to select multiresistant bacteria, the medium was supplemented with three antibiotics (40 U/mL polymyxin B, 16 μg/mL nalidixic acid (NA), 20 μg/mL vancomycin (VA)), an antifungal (4 μg/mL amphotericin B), and an antiseptic (500 μg/mL cycloheximid).

For each sample, 50 mL of water was concentrated by centrifugation at 6,000 rpm for 20 minutes and the resulting pellets were recovered and inoculated on antimicrobial supplemented BHIA plates. For each 50 mL volume, five

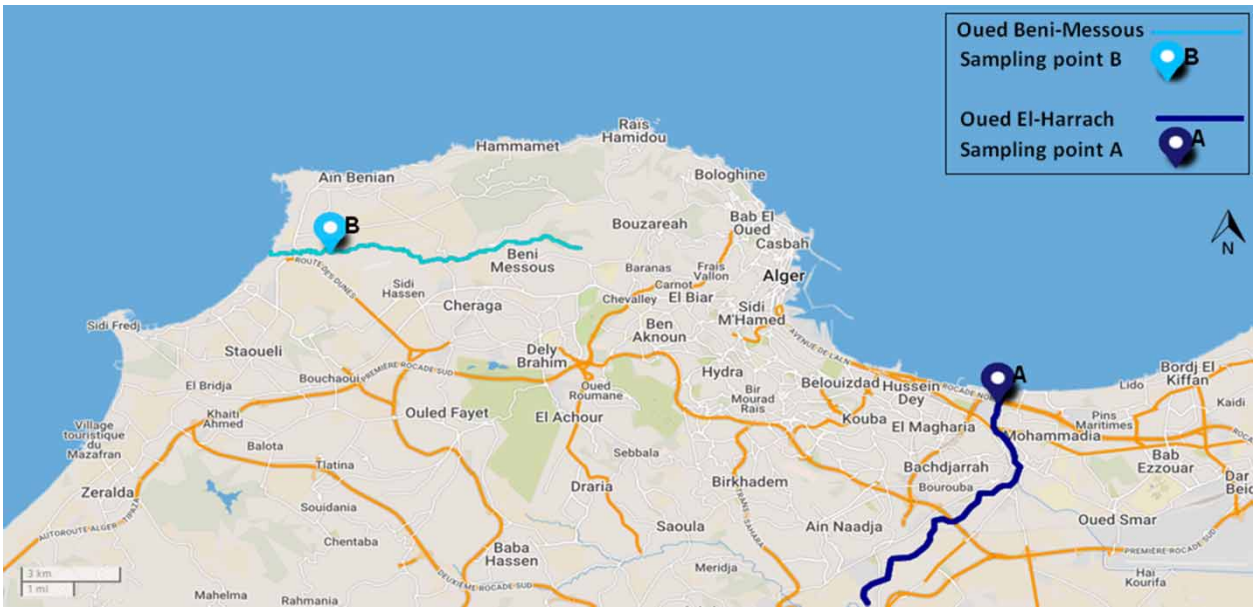

Figure 1 | Geographical locations of the sampling sites in Algiers. A: sampling point in Oued El-Harrach; B: sampling point in Oued Beni-Messous.

different plates were inoculated and the experiment was performed in duplicate. Thus, for each studied river, 20 plates were inoculated and, then, incubated at 30 °C for 24 hours to 10 days.

Bacteria identification

After growth, dominant and morphologically distinct colonies were purified and isolated by repeated transfers on fresh BHIA plates. Colony morphology was observed allowing us to distinguish different strains.

Isolates were analyzed by Gram staining, activities of oxidase, catalase, nitrate reductase, VP-MR test, motility, indole production, citrate utilization, and respiratory type according to *Bergey's Manual of Systematic Bacteriology* (Holt *et al.* 1994).

Aerobic and anaerobic Gram-negative rods were identified by API 20 NE and API 20 E galleries, respectively (API system, Biomerieux, France).

Strains presenting particular macroscopic or microscopic characteristics and which were not stained by Gram technique were tested for Zhiel–Neelsen staining, which was performed from colony fragments of the isolated subcultures.

For some of the strains, a confirmative molecular identification was carried out by 16S rDNA sequence analysis. DNA purification, polymerase chain reaction (PCR) amplification and sequencing of the 16S rRNA gene were performed as described previously (Ben DhiaThabet *et al.* 2004).

Phylogenetic analysis

Molecular Evolutionary Genetics Analysis (MEGA) software, version 6.0, was used to assist the phylogenetic analyses and the phylogenetic trees' construction (Tamura *et al.* 2013). Similar 16S rRNA gene sequences for study of the strains were obtained by using Eztaxon (Kim *et al.* 2012). Multiple alignments of data were performed by CLUSTAL W (Thompson *et al.* 1994). Evolutionary distances were calculated by using maximum composite likelihood method and are in the units of the number of base substitutions per site (Tamura *et al.* 2004). The phylogenetic trees were reconstructed with the neighbor-joining algorithm (Saitou & Nei 1987). Topology of the resultant trees was evaluated by bootstrap analyses of the neighbor-joining dataset, based on 1,000 resamplings.

Determination of antibiotic resistance

Disk diffusion method on Mueller Hinton agar plates (Difco) was used to test the resistance or sensitivity of the bacterial strains towards 34 antibiotics. The following antibiotic disks were used (μg or International Unit 'IU'/disk): amoxicillin + clavulanic acid (AMC) 30 μg, ampicillin (AMP) 10 μg, amoxicillin (AMX) 25 μg, amikacin (AK) 30 μg, bacitracin (B) 10IU, chloramphenicol (C) 30 μg, ceftazidime (CAZ) 30 μg, cefixime (CFM) 5 μg, cefoperazone (CFP) 75 μg, cephalotin (CH) 30 μg, ciprofloxacin (CIP) 5 μg, cefaclor (CJ) 30 μg, colistin (CS) 10 μg, cefotaxime (CTX) 30 μg, cefazolin (CZ) 30 μg, erythromycin (E) 15 μg, cephoxitin (FOX) 30 μg, imipenem (IMP) 10 μg, lincomycin (L) 15 μg, mezlocillin (MZ) 75 μg, neomycin (N) 30 IU, NA 30 μg, nitroxoline (NI) 20 μg, novobiocin (NV) 30 μg, oxacillin (OX) 5 μg, penicillin (P) 10 IU, pipemedic acid (PI) 20 μg, pristinamycin (PT) 15 μg, streptomycin (S) 10 μg, spiramycin (SP) 100 μg, sulphonamid (SSS) 300 μg, tetracycline (TE) 30 μg, VA 30 μg, and virginamycin (VI) 15 μg (Hi-media, India).

In addition, for the *Mycobacterium* genus, susceptibility was tested for rifampicin (RIF) 5 μg (Hi-media, India).

The results were interpreted according to the guidelines of the Antibiogram Committee of the French Society for Microbiology. *Escherichia coli* ATCC 25922 was used as a control strain for antimicrobial susceptibility testing.

Determination of minimum inhibitory concentrations of heavy metals

For each strain, the minimum inhibitory concentrations (MICs) of heavy metals were determined by spot plate method as described by Malik & Aleem (2011). The metals Hg^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Cr^{6+}, Co^{2+}, Zn^{2+}, and Li^{+}, used as $HgCl_2$, $PbCl_2$, $CdCl_2$, $CuCl_2.2H_2O$, $K_2Cr_2O_7$, $CoCl_2$, $ZnCl_2$, and LiCl, were added to Mueller Hinton agar at varying concentrations, ranging from 12.5 to 3,000 μg/mL. The plates were incubated at 30 °C for 48 hours to 10 days.

The MIC was defined as the minimum concentration of heavy metal which inhibits the growth of the tested strain, and the resistant thresholds for heavy metals were set as: 12.5 μg/mL for mercury, 200 μg/mL for cadmium and copper, and 100 μg/mL for zinc, lead, chrome, cobalt, and lithium (Malik & Aleem 2011).

Plasmid curing

The most antibiotic and heavy metal resistant strain of each sample was selected and submitted to plasmid curing in order to determine the location of the resistance genes. Overnight cultures of the strains were grown in Luria-Bertani broth (Difco) supplemented with 100 µg/mL of acridine orange dye, set as the sub-inhibitory concentration which can inhibit plasmids' replication. A control tube lacking curing agent was also included in the experiment. The tubes were incubated at 30 °C for 24 hours. Contents of the tubes were then plated on nutrient agar.

After incubation, 300 cured derivatives were recovered with sterile toothpicks and transferred onto nutrient agar plates, at the rate of 52 clones per plate, as shown in Figure 2. Each cured derivative along with its parental strain was then subcultured in selective media supplemented with the previously tested antibiotics and heavy metals. A complete antibiogram was carried out for the cured derivatives that did not grow on selective media.

Isolation of plasmid DNA

The plasmid DNA was extracted as described by Birnboim & Doly (1979) and visualized through agarose gel electrophoresis according to standard procedure. Electrophoresis was carried out at 80 V for 3 hours on a 0.8% agarose gel. 1 kb plus DNA ladder and λ DNA digested with EcoRI and HindIII were used as standard markers.

RESULTS

Physicochemical characteristics and heavy metal concentrations

The physicochemical characteristics and the heavy metal concentrations measured in Oued El-Harrach and Oued Beni-Messous are presented in Table 1.

Isolation and identification of multiresistant opportunistic bacteria

The selective isolation of multiresistant opportunistic bacteria, from the two river waters, was carried out on a rich medium (BHIA) supplemented with three antibiotics, an antifungal, and an antiseptic. After the incubation period, 11 and 10 different strains were isolated from Oued El-Harrach and Oued Beni-Messous samples, respectively.

Morphological characteristics were studied showing 18/21 rod-shaped Gram-negative strains. A study of biochemical characteristics allowed us to distinguish two principal types in these strains: fermentative bacteria for which the identification was performed by Api 20 E gallery and non-fermentative bacteria identified by API 20 NE gallery.

Identification and characteristics of the 21 strains isolated from Oued El-Harrach and Oued Beni-Messous rivers are summarized in Table 2 and the macroscopic features of some of them are illustrated in Figure 3.

The most represented genera were *Aeromonas* and *Escherichia,* each genus counting three strains, followed by

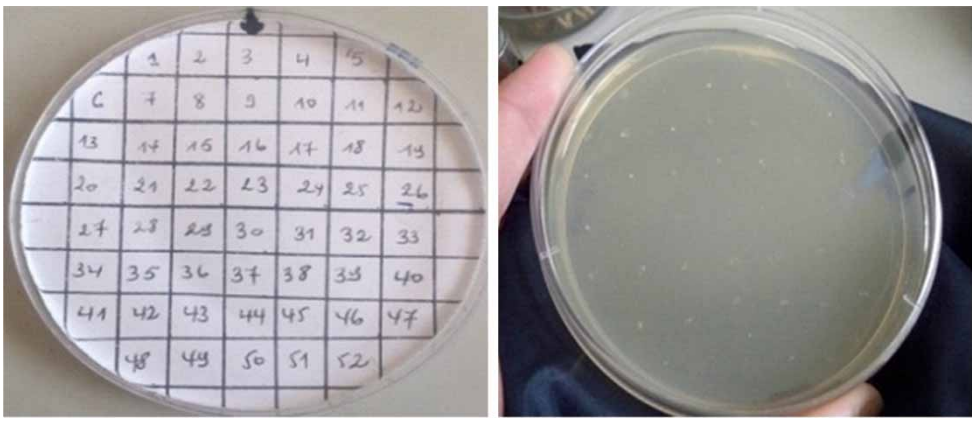

Figure 2 | Disposition for recovered cured derivatives on a Petri dish.

Table 1 | Temperature, pH, and heavy metal concentrations in Oued El-Harrach and Oued Beni-Messous

Characteristics	Oued El-Harrach	Oued Beni-Messous
Temperature (°C)	19	18
pH	7.82	7.46
Cadmium (μg/L)	5.8	6.3
Copper (μg/L)	24	37
Lead (μg/L)	43	84

Acinetobacter, *Pseudomonas*, and *Bordetella* (two strains, each). Other rod-shaped Gram-negative genera (represented by one strain, each) were *Klebsiella*, *Proteus*, *Citrobacter*, *Enterobacter*, *Pasteurella*, and *Brevundimonas*.

Strain PA1 presented yellow colonies and Gram-positive cocci arranged in tetrads. It was found to be catalase and oxidase positive. These characteristics allowed us to pre-identify the strain as belonging to the genus *Micrococcus*.

Strain TA5 was slightly stained by Gram technique and presented inlaid orange colonies with a woolly appearance. The microscopic observation showed lightly Gram-positive, long, filamentous and tangled bacilli. These specifications suggested that the strain belonged to the *Actinobacteria* group.

Strain SA11 was not stained at all by Gram technique and formed rough, friable, and cauliflower-shaped colonies in isolated cultures.

On the basis of these morphological characteristics, a Zhiel–Neelsen staining was done for TA5 and SA11 strains. Strain TA5 was partially stained while strain SA11 responded positively and showed pink-colored long bacilli, dispersed or forming irregular clumps, suggesting that the latter belonged to the genus *Mycobacterium* (Figure 4).

Confirmation of the taxonomical statutes by 16S DNA sequence analysis was done for 14 strains. The results are shown in Table 1. The 16S rRNA sequences for strains S1, S4, S10, R2, L4, S5, T8, HBA2, P3, P5, P18, TA5, PA1 and SA11 have been submitted to the NCBI Genbank database under the following accession numbers:

S1: KP233464; S4: KP233465; S10: KP233466; R2: KP233467; L4: KM603199; S5: KP233468;
T8: KP233469; HBA2: KP402409; P3: KP402410; P5: KP402411; P18: KP402412; TA5: KP402413;
PA1: KP402414; SA11: KP402415.

A phylogenetic analysis was carried out, and the resulting phylogenetic trees are shown in Figure 5.

Determination of antibiotic resistance

An antibiogram was carried out for the 21 isolated strains against 34 molecules representing 13 different antibiotic families. The antibiotic resistance profiles are presented in Table 1 and the resistance frequencies are shown in Table 3.

No significant differences between resistance frequencies in Oued El-Harrach and Oued Beni-Messous isolates were noticed (Table 3).

A high level of resistance was observed against β-lactam antibiotics. Indeed, P had the highest resistance frequency (20/21 strains) followed by OX (19/21), AMP and AMX (18/21). As well, 18 strains were resistant to CH (first-generation cephalosporin), whereas 17 and 16 strains were resistant to CAZ (third-generation cephalosporin) and CJ (second-generation cephalosporin), respectively. However, no resistance was found against IMP.

In the quinolones family, a high resistance frequency was noted for NA (20/21) whereas 12 and 6 strains were resistant to PI and CIP, respectively.

Nineteen isolates were L resistant (lincosamides) and 17 were VA resistant (glycopeptides) while 18 and 12 strains were resistant to B and CS (polypeptides), respectively.

Aminosides, macrolides, TEs, streptogramins, aminocoumarins, phenicols, and SSSs showed lower resistance frequencies (ranging from 1 to 11 resistant strains).

None of our strain was resistant to NI (nitroquinolines).

As the strain SA11 was confirmed to belong to the genus *Mycobacterium* by molecular tools, its sensibility was tested against RIF. The strain was RIF resistant.

All isolates exhibited resistance to multiple antibiotics with a minimum of 7 and a maximum of 25 antibiotics at the same time (Table 4). The most represented patterns were 19 and 23 resistances with 3/21 strains, each.

Determination of heavy metal resistance

For the 21 isolates, the MICs for Hg^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Cr^{6+}, Co^{2+}, Zn^{2+}, and Li^{+} were determined by spot plate method. The MICs for each heavy metal are presented in Table 1 and the resistance patterns are shown in Table 5.

Table 2 | Strains isolated from Oued-El-Harrach and Oued Beni-Messous and their characterization

Water sample	Isolate	Biochemical identification	Molecular identification (16S rRNA similarity)	Antibiotic resistance	Heavy metals MICs
Oued El-Harrach	S1	*Klebsiella* sp.	*Klebsiella oxytoca* (98%)	AMX, AMP, P, OX, MZ, CH, CFP, NA, B, PT, VI, VA, SSS, L	Co_{2000}, Li_{3000}, Zn_{3000}, Cu_{2000}, Hg_{100}, Cr_{1000}, Cd_{1000}, Pb_{3000}
	S3	*Aeromonas* sp.	ND	AMX, AMP, P, AMC, OX, MZ, CH, CJ, CFP, CAZ, NA, SP, B, CS, PT, NV, C, VA, SSS, L	Co_{1000}, Li_{3000}, Zn_{500}, Cu_{500}, Hg_{50}, Cr_{500}, Cd_{1000}, Pb_{500}
	S4	*Escherichia coli*	*Escherichia coli* (99%)	AMX, AMP, P, AMC, OX, MZ, CH, CJ, CFP CAZ, CIP, NA, PI, E, SP, B, VI, VA, L.	Co_{2000}, Li_{3000}, Zn_{3000}, Cu_{1000}, Hg_{100}, Cr_{1000}, Cd_{1000}, Pb_{3000}
	S10	*Proteus* sp.	*Proteus mirabilis* (99%)	AMX, AMP, P, OX CH, CJ, CFP, CAZ, NA, PI, E, TE, B, CS, PT, VI, NV, C, VA, SSS	Co_{3000}, Li_{3000}, Zn_{3000}, Cu_{3000}, Hg_{100}, Cr_{500}, Cd_{1000}, Pb_{3000}
	R2	*Bordetella* sp.	*Bordetella bronchiseptica* (97%)	AMX, AMP, P, OX, CH, CZ, CJ, CFM, CFP, CAZ, CTX, NA, PI, AK, S, B, CS, L, VA	Co_{1000}, Li_{3000}, Zn_{1000}, Cu_{1000}, Hg_{100}, Cr_{50}, Cd_{50}, Pb_{3000}
	R3	*Pseudomonas* sp.	ND	AMP, P, OX, CH, CZ, CJ, CFM, CFP, FOX, CAZ, NA, PI, B, CS, L	Co_{500}, Li_{3000}, Zn_{1000}, Cu_{1000}, Hg_{100}, Cr_{100}, Cd_{50}, Pb_{1000}
	L4	*Acinetobacter* sp.	*Acinetobacter* sp. (97%)	AMX, AMP, P, OX, MZ, CH, CJ, CFP, CAZ, CTX, CIP, NA, PI, S, E, SP, TE, B, CS, PT, VI, NV, VA, SSS, L	Co_{1000}, Li_{3000}, Zn_{3000}, Cu_{3000}, Hg_{100}, Cr_{1000}, Cd_{1000}, Pb_{3000}
	P7	*Escherichia coli*	ND	CFP, NA, PI, NV, C, VA, L	Co_{1000}, Li_{2000}, Zn_{1000}, Cu_{1000}, Hg_{100}, Cr_{1000}, Cd_{50}, Pb_{3000}
	P17	*Pasteurella* sp.	ND	AMX, P, OX, MZ, CH, CZ, CJ, CFM, FOX, CAZ, CTX, CIP, NA	Co_{1000}, Li_{50}, Zn_{1000}, Cu_{1000}, Hg_{50}, Cr_{1000}, Cd_{100}, Pb_{3000}
	S5	*Escherichia coli*	*Escherichia coli* (99%)	AMX, AMP, P, AMC, OX, MZ, CH, CJ, CAZ, CIP, NA, E, TE, B, CS, PT, VI, NV, VA, SSS, L	Co_{1000}, Li_{3000}, Zn_{3000}, Cu_{1000}, Hg_{50}, Cr_{1000}, Cd_{1000}, Pb_{3000}
	TA5	*Actinobacteria*	*Nocardia farcinica* (98%)	AMX, AMP, P, AMC, OX, MZ, CH, CIP, NA, SP, B, CS, PT, VI, VA, SSS, L	Co_{1000}, Li_{1000}, Zn_{1000}, Cu_{1000}, Hg_{100}, Cr_{1000}, Cd_{1000}, Pb_{1000}
Oued Beni-Messous	T8	*Enterobacter* sp.	*Enterobacter* sp. (99%)	AMX, AMP, P, AMC, OX, MZ, CH, CZ, CFP, CJ, CAZ, CIP, NA, PI, S, SP, TE, B, CS, VI, NV, VA, L	Co_{2000}, Li_{3000}, Zn_{2000}, Cu_{100}, Hg_{100}, Cr_{1000}, Cd_{1000}, Pb_{3000}
	P4	*Acinetobacter* sp.	ND	AMX, AMP, P, AMC OX, MZ, CH, CJ, CFP, CAZ, NA, PI, B, VA, L	Co_{1000}, Li_{3000}, Zn_{1000}, Cu_{1000}, Hg_{100}, Cr_{1000}, Cd_{1000}, Pb_{1000}
	HBA2	*Brevundimonas* sp.	*Brevundimonas vancanneytii* (97%)	AMX, AMP, P, AMC, OX, MZ, CH, CZ, CJ, CAZ, NA, S, CS, VI, NV, VA, L	Co_{1000}, Li_{3000}, Zn_{2000}, Cu_{1000}, Hg_{100}, Cr_{1000}, Cd_{1000}, Pb_{3000}
	P3	*Aeromonas* sp.	*Aeromonas hydrophila* (99%)	AMX, AMP, P, AMC, OX, MZ, CH, CZ, CJ, CFM, FOX, CAZ, CTX, NA, E, SP, B, PT, VI, NV, C, VA, L	Co_{1000}, Li_{3000}, Zn_{1000}, Cu_{1000}, Hg_{50}, Cr_{1000}, Cd_{100}, Pb_{1000}
	P5	*Pseudomonas* sp.	*Pseudomonas fluorescens* (99%)	AMX, AMP, P, AMC, OX, MZ, CH, CZ, CJ, CFP, FOX, CAZ, NA, PI, SP, TE, B, CS, PT, NV, VA, L	Co_{1000}, Li_{2000}, Zn_{500}, Cu_{500}, Hg_{100}, Cr_{1000}, Cd_{100}, Pb_{1000}
	P12	*Aeromonas* sp.	ND	AMX, AMP, P, AMC, OX, MZ, CH, CZ, CJ, CFP, CAZ, NA, PI, E, SP, B, PT, C, VA, SSS, L	Co_{1000}, Li_{3000}, Zn_{3000}, Cu_{2000}, Hg_{100}, Cr_{1000}, Cd_{1000}, Pb_{3000}

(*continued*)

Table 2 | continued

Water sample	Isolate	Biochemical identification	Molecular identification (16S rRNA similarity)	Antibiotic resistance	Heavy metals MICs
	P16	*Bordetella* sp.	ND	AMX, AMP, P, AMC, OX, CJ, CZ, CFM, CFP, FOX, CAZ, NA, PI, S, N, E, SP, TE, B, VI, C, VA, SSS, L	Co_{2000}, Li_{3000}, Zn_{1000}, Cu_{1000}, Hg_{100}, Cr_{1000}, Cd_{1000}, Pb_{3000}
	P18	*Citrobacter* sp.	*Citrobacter freundii* (99%)	AMX, AMP, P, AMC, OX, CH, CZ, CJ, CFP, FOX, CAZ, NA, PI, N, E, SP, TE, B, CS, PT, VI, NV, VA, SSS, L	Co_{2000}, Li_{3000}, Zn_{3000}, Cu_{3000}, Hg_{100}, Cr_{1000}, Cd_{1000}, Pb_{3000}
	PA1	*Micrococcus* sp.	*Micrococcus luteus* (100%)	P, CFP, CAZ, NA, E, B, L	Co_{1000}, Li_{3000}, Zn_{1000}, Cu_{2000}, Hg_{50}, Cr_{1000}, Cd_{1000}, Pb_{3000}
	SA11	ND	*Mycobacterium fortuitum* (98%)	AMX, AMP, P, AMC, OX, MZ, CH, SP, B, CS, PT, SSS, L, RIF	Co_{2000}, Li_{3000}, Zn_{1000}, Cu_{1000}, Hg_{100}, Cr_{1000}, Cd_{1000}, Pb_{1000}

ND, Not determined.

Figure 3 | Colonies of (a) strain L4, (b) strain SA11, (c) strain P18 on BHI agar plates.

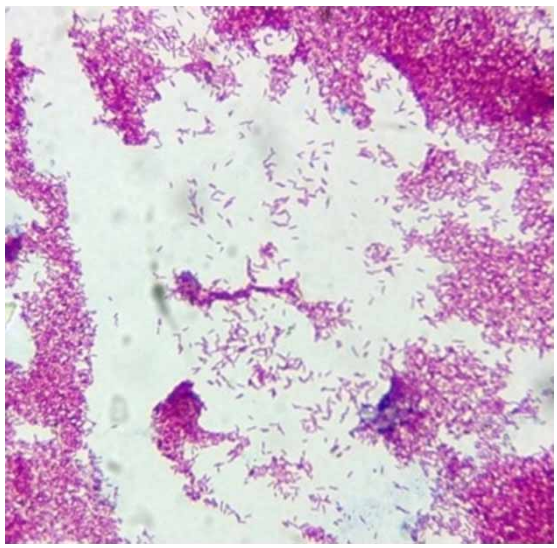

Figure 4 | Microscopic observation of positive Ziehl–Neelsen staining of strain SA11 (Photonic microscope, ×100).

As for antibiotics, no significant differences between resistance frequencies in Oued El-Harrach and Oued Beni-Messous isolates were noted. All strains were resistant to mercury, lead, cobalt, and zinc whereas copper and lithium both counted 20/21 resistant strains. Chromium and cadmium had the lowest resistance frequencies with 19/21 and 15/21 strains, respectively.

In addition, all of the strains showed multiple heavy metal resistance with a minimum of 6 resistances at the same time. Indeed, 14 strains were resistant to all 8 heavy metals tested. Most of them were not only resistant but displayed relatively high MICs for all heavy metals, considering their resistance ranges: for lithium, 17 strains were inhibited at MIC of 3,000 μg/mL while for lead 14 strains were inhibited at MIC of 1,000 μg/mL (Table 5).

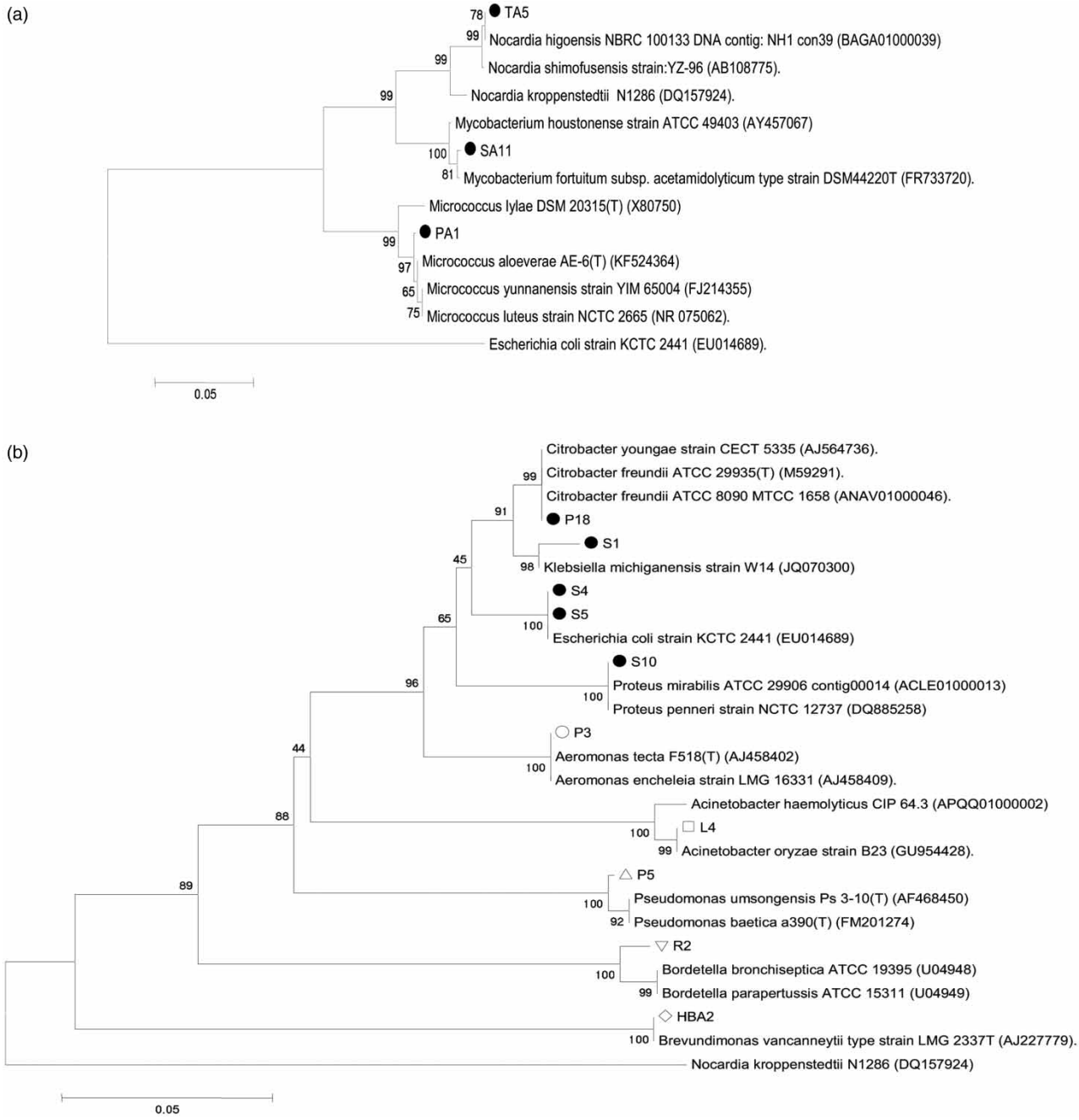

Figure 5 | Phylogenetical relationships between Oued El-Harrach and Oued Beni-Messous selected strains and the most related type strains' species using partial 16S rRNA sequences: (a) Gram-positive bacteria; (b) Gram-negative bacteria. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. Tree topology was constructed using MEGA 6.0. Bootstrap values (1,000 replicates were indicated at the nodes). *Escherichia coli* KCTC2441 and *Nocardia kroppenstedtii* N1286 sequences were added as an outgroup for these trees, respectively.

Plasmid curing and plasmid profile analysis

Acinetobacter sp. strain L4 and *Citrobacter freundii* strain P18 showed the highest levels of resistance against heavy metals and antibiotics. Both strains were resistant to 25 antibiotics and to all of the 8 heavy metals tested with high MICs (ranging from 50 to 100 μg/mL for mercury and from 100 to 3,000 for the other metals). In order to

Table 3 | Antibiotic resistance frequencies in Oued El-Harrach and Oued Beni-Messous isolates

		Number of resistant isolates		
Antibiotic/Drug		**Oued El-Harrach (*N* = 11)**	**Oued Beni-Messous (*N* = 10)**	**Total (*N* = 21)**
β-Lactams	AMX	9	9	18
	AMP	9	9	18
	P	10	10	20
	AMC	4	9	13
	OX	10	9	19
	MZ	7	7	14
	CH	10	8	18
	CZ	3	7	10
	CJ	8	8	16
	CFM	3	2	5
	CFP	8	7	15
	FOX	2	4	6
	CAZ	8	9	17
	CTX	3	1	4
	IMP	0	0	0
Quinolones	CIP	4	2	6
	NA	11	9	20
	PI	6	6	12
Aminosides	AK	1	0	1
	S	2	3	5
	N	0	2	2
Macrolides	E	4	5	9
	SP	4	7	11
TEs	TE	3	4	7
Polypeptides	B	9	9	18
	CS	7	5	12
Streptogramins	PT	6	5	11
	VI	6	5	11
Aminocoumarin	NV	5	5	10
Phenicols	C	3	3	6
Nitroquinolines	NI	0	0	0
Glycopeptides	VA	9	8	17
Sulfonamides	Sulfonamide (SSS)	6	4	10
Lincosamides	L	9	10	19

determine if resistance genes were chromosome or plasmid encoded, strains L4 and P18 were submitted to plasmid curing. For each strain, 300 cured derivatives were tested for antibiotic and heavy metal resistance.

For strain P18, out of 300 recovered colonies, one cured derivative showed susceptibility to zinc, lead and TE. A curing frequency of 3×10^{-2} was thus calculated. The plasmid profile analysis demonstrated that strain P18 harbored a high molecular weight plasmid lost by the cured variant (Figure 6, lanes B and D).

However, none of the 300 cured derivatives of strain L4 showed any change in their multiple antibiotic and heavy metal resistance. The plasmid profile analysis revealed no loss of any plasmid compared to the parental strain (Figure 6, lanes A and C).

DISCUSSION

The physicochemical analysis of Oued El-Harrach and Oued Beni-Messous showed that both sites contained cadmium, copper, and lead. All the measured concentrations were higher than the thresholds defined by the European and French standards for surface waters (Directive 2013/39/UE; Order of 27 July 2015: JORF n°0198).

Out of the 21 multiresistant isolates, 18 strains were Gram-negative rods, representing 11 different genera. Bacteria belonging to the genera *Aeromonas, Escherichia, Acinetobacter, Pseudomonas, Bordetella, Klebsiella, Proteus, Citrobacter, Enterobacter, Pasteurella*, and *Brevundimonas* were isolated and identified by biochemical and molecular approaches. These genera are widely distributed in the environment and known as opportunistic bacteria causing severe infections in immunodepressed subjects (Cabrera *et al.* 2011). Analogous studies have shown a similar prevalence of multiresistant Enterobacteria and non-fermentative Gram-negative rods in polluted environments (Allouache *et al.* 2012; Akkan *et al.* 2013).

Moreover, three Gram-positive strains were isolated from the two water samples. Taxonomical status of strains PA1, TA5, and SA11 were confirmed by 16S RNA gene sequencing showing that they belonged to the genera *Micrococcus*, *Nocardia*, and *Mycobacterium*, respectively. These genera are also incriminated in opportunistic infections and known as natural inhabitants of the environment (Falkinham 1996; Laurent *et al.* 1999; Kao *et al.* 2012).

Strain SA11 was identified as belonging to the species *Mycobacterium fortuitum*. NTM are causal agents of severe opportunistic infections and it has been demonstrated that their transmission occurs through the environment (Falkinham 1996; Giulieri *et al.* 2011). To the best of our knowledge, this is the first study in Algeria to report the detection of a

Table 4 | Pattern of antibiotic resistance in Oued El-Harrach and Oued Beni-Messous isolates

Number of antibiotics	Number of resistant strains	Resistance pattern
7	2	CFP, NA, PI, NV, C, VA, L P, CFP, CAZ, NA, E, B, L
12	1	AMX, P, OX, MZ, CH, CZ, CJ, CFM, FOX, CAZ, CTX, CIP, NA
14	2	AMX, AMP, P, OX, MZ, CH, CFP, NA, B, PT, VI, VA, SSS, L AMX, AMP, P, AMC, OX, MZ, CH, SP, B, CS, PT, SSS, L, RIF
15	2	AMP, P, OX, CH, CZ, CJ, CFM, CFP, FOX, CAZ, NA, PI, B, CS, L AMX, AMP, P, AMC OX, MZ, CH, CJ, CFP, CAZ, NA, PI, B, VA, L
17	1	AMX, AMP, P, AMC, OX, MZ, CH, CZ, CJ, CAZ, NA, S, CS, VI, NV, VA, L
18	1	AMX, AMP, P, AMC, OX, MZ, CH, CIP, NA, SP, B, CS, PT, VI, VA, SSS, L
19	3	AMX, AMP, P, AMC, OX, MZ, CH, CJ, CFP CAZ, CIP, NA, PI, E, SP, B, VI, VA, L AMX, AMP, P, AMC, OX, MZ, CH, CJ, CFP, CAZ, NA, SP, B, CS, PT, NV, C, VA, SSS, L AMX, AMP, P, OX, CH, CZ, CJ, CFM, CFP, CAZ, CTX, NA, PI, AK, S, B, CS, L, VA
20	2	AMX, AMP, P, OX CH, CJ, CFP, CAZ, NA, PI, E, TE, B, CS, PT, VI, NV, C, VA, SSS AMX, AMP, P, AMC, OX, MZ, CH, CZ, CJ, CFP, CAZ, NA, PI, E, SP, B, PT, C, VA, SSS, L
21	2	AMX, AMP, P, AMC, OX, MZ, CH, CJ, CAZ, CIP, NA, E, TE, B, CS, PT, VI, NV, VA, SSS, L
22	1	AMX, AMP, P, AMC, OX, MZ, CH, CZ, CJ, CFP, FOX, CAZ, NA, PI, SP, TE, B, CS, PT, NV, VA, L
23	3	AMX, AMP, P, AMC, OX, MZ, CH, CZ, CFP, CJ, CAZ, CIP, NA, PI, S, SP, TE, B, CS, VI, NV, VA, L AMX, AMP, P, AMC, OX, MZ, CH, CZ, CJ, CFM, FOX, CAZ, CTX, NA, E, SP, B, PT, VI, NV, C, VA, L AMX, AMP, P, AMC, OX, CJ, CZ, CFM, CFP, FOX, CAZ, NA, PI, S, N, E, SP, TE, B, VI, C, VA, SSS, L
25	2	AMX, AMP, P, OX, MZ, CH, CJ, CFP, CAZ, CTX, CIP, NA, PI, S, E, SP, TE, B, CS, PT, VI, NV, VA, SSS, L AMX, AMP, P, AMC, OX, CH, CZ, CJ, CFP, FOX, CAZ, NA, PI, N, E, SP, TE, B, CS, PT, VI, NV, VA, SSS, L

Table 5 | Pattern of heavy metal resistance in strains isolated from Oued El-Harrach and Oued Beni-Messous

Number of metals	Number of resistant strains	Resistance pattern
6	2	Hg^{2+}, Pb^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Li^{+}
	1	Hg^{2+}, Pb^{2+}, Cu^{2+}, Cr^{6+}, Co^{2+}, Zn^{2+}
7	3	Hg^{2+}, Pb^{2+}, Cu^{2+}, $Cr^{6+,}$ Co^{2+}, Zn^{2+}, Li^{+}
	1	Hg^{2+}, Pb^{2+}, Cd^{2+}, $Cr^{6+,}$ Co^{2+}, Zn^{2+}, Li^{+}
8	14	Hg^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, $Cr^{6+,}$ Co^{2+}, Zn^{2+}, Li^{+}

NTM in the environment. Indeed, Algerian studies report the isolation of clinical NTM strains from sputum of immunodepressed patients (Natéche 2007) and an animal-associated NTM new species from a goat lung lesion (Sahraoui *et al.* 2011). Nonetheless, this would be the first time that NTM were recovered from an environmental source in Algeria.

In this work, all the isolated strains showed multiple antibiotics resistance with a minimum of seven resistances at the same time. Both studied rivers receive hospital effluents that may contain antimicrobial residues. It has been reported that wastewater discharges from hospitals were associated with an increased prevalence of antibiotic resistance (Elmanama *et al.* 2006). Furthermore, even exposure to low concentrations of antimicrobial agents over long periods of time may result in selection and consequent spread of resistance to antibiotics (Chen *et al.* 2015).

Hsu *et al.* (1992) pointed out that this remarkable ability of bacteria to resist various antibiotics might reflect the history of antibiotic applications, thus allowing bacterial resistance to be used as an indicator of antibiotic application.

In our study, except for IMP for which all the strains were sensitive, the β-lactams family had the highest resistance frequencies. These antibiotics, possessing a broad spectrum of activity, are commonly prescribed in the therapeutic scheme of various infections, especially for those involving Gram-negative rods. However, it is reported that these bacteria are increasingly resistant to these molecules, mainly by β-lactamase

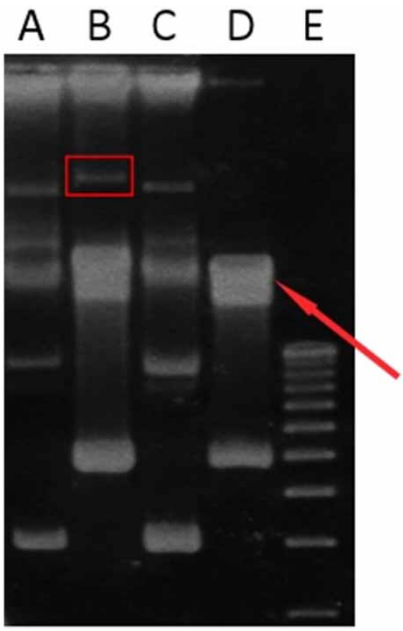

Figure 6 | Agarose gel profiles of plasmid DNA isolated from strains L4 and P18. Lane A: *Acinetobacter* sp. (L4) parental strain; lane B: *Citrobacter freundii* P18 parental strain; lane C: L4 cured derivative; lane D: P18 cured derivative; lane E: 1 kb plus DNA ladder. The rectangle indicates the plasmid in P18 parental strain and the arrow indicates the chromosome residues.

enzyme production encoded by transferable genetic elements (Riccio *et al*. 2000). IMP is a carbapenem antibiotic known for its high resistance to the β-lactamase enzymes and plays a key role in the treatment of infections which cannot be treated with other antibiotics. Thus, IMP is only prescribed in the case of multidrug-resistant (MDR) bacterial infections in seriously affected inpatients. This could explain the fact why none of our strains were resistant to this molecule.

Out of the 21 isolated strains, 12 were resistant to CS. The emergence of this resistance could lead to a major issue in the treatment of MDR Gram-negative bacteria-associated infections. CS has, indeed, appeared as a key treatment in such infections during the past few years. For instance, the New-Delhi metallo-β-lactamase (NDM-1), a carbapenemase that allows resistance to a broad range of antibiotics, has been described in Gram-negative bacteria. Most of the time, the NDM-1 positive bacteria remained susceptible to CS (Nordmann *et al*. 2011). Also, it has recently been reported that CS was one of the most effective antibiotics for removal of biofilm producers in indwelling medical devices (Mishra *et al*. 2015). Therefore, the potential dissemination of CS resistance genes by horizontal transfers represents an important public health matter.

In this work, VA also had a high resistance frequency since the isolation was made on a VA supplemented medium. Particular attention should be given to VA-resistant bacteria, knowing that this molecule is prescribed in the case of methicillin-resistant *Staphylococcus aureus*, one of the most feared nosocomial pathogen (Chambers & DeLeo 2009). Here again, resistance genes could be transferred among bacteria and lead to the spread of VA resistance.

It is to be noted that strain SA11, belonging to the genus *Mycobacterium*, was found to be resistant to RIF. This antibiotic is one of the most commonly prescribed for *Mycobacterium* infections. Thus, this resistance could lead to failures in the treatment of NTM infections.

More generally, genes involved in resistance mechanisms are often carried by mobile elements, such as plasmids and integrons that can be transferred among a broad phylogenetic range of bacteria. It has been demonstrated that these horizontal transfers in the environment contribute to the evolution of the bacterial resistomes and to the emergence of antibiotic resistance (Amos *et al*. 2014).

The 21 isolated strains showed high levels of heavy metal resistance. Most of them displayed resistance to all of the tested metals (14/21) with relatively high MICs. Our results are comparable to those reported in similar studies (Malik & Aleem 2011; Akkan *et al*. 2013) which indicate bacterial heavy metal resistance in polluted waters and explain that chemical pollution of these environments could constitute a selective pressure leading to the selection of metal resistance genes in bacterial communities. Moreover, they report combined resistance to heavy metals and antibiotics and suggest a probable co-location of the implied genes in the same plasmid or integron which are, thus, more likely to be transferred together among bacteria.

As well, it has been suggested that resistance mechanisms involved in antibiotic resistance were also involved in metal resistance. Indeed, an efflux pump that can extrude both antibiotics and heavy metals has been described by Mata *et al*. (2000). This implies that the presence of heavy metals in the environment is sufficient to select resistance genes involved in both antibiotic and heavy metal resistances. Thereby, the fact that Oued Beni-Messious and Oued El-Harrach contain heavy metals suggests that this contamination constitutes a selective pressure that could have led to the bacterial multiresistance determined in this study.

Acinetobacter sp. strain L4 and *Citrobacter freundii* strain P18 were selected for their high levels of resistance and submitted to plasmid curing in order to determine their resistance determinants. After the curing process, strain L4 did not show any change in its resistance abilities and its plasmid profile analysis revealed no loss of plasmid DNA compared to the parental strain. From these observations, we concluded that either the multiple antibiotics and heavy metals resistance was chromosome encoded or the curing process did not target the aimed markers. In contrast, one cured derivative of strain P18 lost its resistance towards TE, zinc, and lead. The plasmid profile analysis showed that the cured variant was lacking a high molecular weight plasmid. We deduced that genes encoding for resistance to zinc, lead, and TE were encoded by the same plasmid.

These results strongly suggest the existence of a correlation between heavy metal and antibiotic resistance encoded by genes that are located in the same genetic structure and can, thus, be transferred together. Otherwise, if a bacterium acquires this plasmid, it will acquire resistance to zinc, lead, and TE at the same time.

Plasmids are known to be the ideal vehicles for recruitment and dissemination of resistance genes because of their ability to be transferred among bacteria, even when they are not phylogenetically related. It is now well known that this phenomenon has greatly contributed to the spread of antibiotic resistance among clinical and environmental bacteria. It has to be noted that plasmid can not only carry resistance genes but also code for virulence factors, conferring pathogenicity to the microorganisms. Thus, when bacteria acquire plasmids they acquire more than one characteristic implied in their danger for human beings.

CONCLUSIONS

This study proves that Oued El-Harrach and Oued Beni-Messous, which contain copper, lead, and cadmium, shelter opportunistic bacterial communities presenting an important rate of resistance to antibiotics and heavy metals. As well, for the first time in Algeria, a multiresistant NTM strain has been recovered from an environmental source. These findings suggest that these environments can constitute a potential path of transmission for opportunistic infections.

Both the rivers studied receive hospital and industry effluents that could have led to the presence of antibiotics and heavy metal residues. This chemical pollution has, necessarily, an impact on the resistance ability of our strains, which certainly harbor important resistance genes.

Plasmid curing of *Citrobacter freundii* strain P18 showed that three of its resistance determinants were located together in the same plasmid which can be transferred to other bacteria.

Thus, the rivers studied constitute a reservoir for circulating transmissible genes that participate in the emergence of antibiotic resistance and imply consequences on human health. Thereby, health authorities should acknowledge a particular interest in the assessment of this bacterial pollution threat.

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