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Research Article

Heavy metal and antibiotic resistance of *Acinetobacter* spp. isolated from diesel fuel polluted soil

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Abstract: Heavy metals pollution of soil and wastewater is a global problem that threatens the environment as they are not degraded or removed and the potential threat to human health comes from the multiple resistances to heavy metals and antibiotics among bacterial populations. The present study was aimed to isolate and identify multiple metal/antibiotic resistant *Acinetobacter* spp. from diesel fuel polluted soil of Al-Dora, Baghdad, Iraq. Initially, a total of 24 bacterial cultures (coded KNZ–1 to KNZ–24) were isolated and identified up to genus level as *Acinetobacter* by morphological, physiological and biochemical characteristics. Screening of heavy metals resistant *Acinetobacter* were conducted by streaking the isolates on nutrient agar plates supplemented with different concentrations: 10, 25, 50 and 100mg/L of the three heavy metals; Hg^{2+}, Cd^{2+} and Pb^{2+}. Out of 24 isolates, 6 (25%) isolates (KNZ–3, KNZ–5, KNZ–8, KNZ–12, KNZ–16 and KNZ–21) were selected as a multiple heavy metal resistant (MHMR) *Acinetobacter* with maximum tolerable concentrations (MTCs); 100–200mg/L for Hg^{2+}, 300-600mg/L for Cd^{2+} and 100–300mg/L for Pb^{2+}. Antibiotic resistance pattern of the selected MHMR isolates was determined by Kirby-Bauer disc diffusion method against 12 different antibiotics belonging to 7 classes. Out of 6 isolates, 4 isolates were multidrug resistance (MDR) with varying degrees. Among them isolate, KNZ–16 showed a wide range of resistance to all tested antibiotics except Levofloxacin and Imipenem. It was concluded that dual resistant *Acinetobacter* is useful in the bioremediation of environments polluted with heavy metals especially the biodegradation of organic pollutants.

Keywords: *Acinetobacter*, Soil, Multiple metal/Antibiotics resistance.

1. Introduction

Heavy metal pollution is a global environmental problem, as a case involving their contamination of soil and water environments is on the increase and this may probably be as a result of industrial revolution [1, 2]. Soils may become contaminated by the accumulation of heavy metals through emissions from the rapidly expanding industrial areas, leaded gasoline, mine tailings and paints, land application of fertilizers, animal manures, sewage sludge, pesticides, spillage of petrochemicals, and atmospheric deposition [3, 4]. Heavy metal contamination of soil may pose risks and hazards to humans and the ecosystem through direct ingestion or contact with contaminated soil, the food chain, drinking of contaminated groundwater, reduction in food quality and land tenure problems [5]. Prolonged exposure to high concentration of heavy metal or hydrocarbon may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased rate of cancer [6]. Microbial survival in polluted environments depends on intrinsic biochemical and structural properties, physiological and genetic adaptation [7]. Microbes apply various types of resistance mechanisms in response to heavy metals which is responsible for alteration of normal cell physiology leading to development of drug resistance in microorganisms [8]. The heavy metal tolerant bacterial species may serve as an important and cost-effective bioremediation tool for the removal of heavy metals from wastewater and industrial effluents, preventing the contamination of the environment [9]. The genus *Acinetobacter* is known to be involved in biodegradation, leaching and removal of several organic and inorganic wastes [10]. The association between heavy metal tolerance and antibiotic resistance is significant since knowledge of metal resistance may provide useful information on mechanisms of antibiotic

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resistance [11]. Therefore, the present study was aimed to isolate and identify the multiple metal/antibiotic resistant *Acinetobacter* from samples of diesel fuel polluted soil of Al-Dora, Baghdad, Iraq. Also, using different classes of antibiotics to investigate the multidrug resistance of the isolates which exhibit multiple heavy metal resistant.

2. Material and Methods

2.1 Collection of Samples

Eighteen Diesel fuel polluted soil samples were collected from Al-Dora, Baghdad, Iraq, in the month of October 2016. Soil samples were collected in a screw cap sterilized plastic bottles. The samples were kept in an icebox at approximately 4°C. The samples were transported to the laboratory of Microbiology, Department of Biotechnology, Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad for bacteriological analysis within 24 h of collection.

2.2 Preparation of Heavy Metal Stock Solutions

Stock solutions (1000mg/L) of the five heavy metals; Hg^{2+}, Pb^{2+} and Cd^{2+} in the form of their salts $HgCl_2$, $Pb(NO_3)_2$ and $CdCl_2$ respectively were prepared. A weight of each of these heavy metal salts that gave a corresponding 1g of each of the respective heavy metal was weighed and dissolved in 1000ml of deionised water. These were left to stand for 30 min to obtain complete dissolution then sterilized by filtration through 0.22µm membrane filters and stored in sterile flasks in the dark at 4°C for no longer than 1 month.

2.3 Screening for Multiple Heavy Metal Resistant Bacteria

Heavy metals resistant bacteria were recovered from the collected samples on nutrient agar medium supplemented with different concentrations; 10, 25, 50 and 100mg/L of each of the three heavy metals; Hg^{2+}, Pb^{2+} and Cd^{2+} in the form of their salts. The nutrient agar medium was sterilized at 121°C for 15 min and allowed to cool 40 – 45°C then the metals were added to medium before plating. Each collected soil sample was diluted with 9ml of sterile saline then 0.1ml from each dilution was spread on the surface of the agar plates and incubated at 37°C for 48 h. Individual bacterial colonies showing and having different morphological appearance on nutrient agar plates were picked up and purified by repeated streaking on nutrient agar.

2.4 Heavy Metal/Antibiotic Resistant *Acinetobacter* isolates identification

Phenotypic identification and morphological characteristics of the selected isolate were studied. The physiological and biochemical tests used to identify the target isolate were compared to Bergey's Manual of Determinative Bacteriology [12]. The biochemical characteristics of the selected isolate were confirmed using Vitek-2 system (BioMerieux®, France).

2.5 Determination of Maximum Tolerable Concentrations (MTCs)

The maximum tolerable concentration (MTC) of heavy metal was selected as the highest concentration of heavy metal that allows growth after 2 days [13]. The MTCs of the tested heavy metals were determined for the obtained bacterial isolates on Tris-minimal salts (TSM) agar medium [14]. The medium was consisted of (g/L): D-glucose (10), Tris-HCl (6.06), NaCl (4.68), KCl (1.49), NH_4Cl (1.07), Na_2SO_4 (0.43), $MgCl_2.2H_2O$, (0.2), $CaCl_2.2H_2O$ (0.03), pH was adjusted to 7 using HCl. The isolated bacterial cultures were primarily screened for MTCs on TSM agar plates individually supplemented with different concentrations of the tested heavy metals. The tested concentrations were; 25 – 300mg/L for Hg^{2+}, 200 – 750mg/L for Cd^{2+}, 25 – 500mg/L for Pb^{2+}. The plates were incubated at 37°C for 48 h then results were recorded.

The obtained results on TSM agar were confirmed on TSM broth medium. 0.1ml of overnight broth culture (OD620= 0.8) of the isolate was inoculated in 10ml sterile TSM broth supplemented with individual concentration of the metals under study. For measurement the growth of the tested organisms, negative control (culture media containing the same concentration of metals without inoculation) and blank (culture media neither inoculated with bacteria nor heavy metal addition). After 48 h, bacterial growth was measured as optical density values at a wavelength of 620nm using spectrophotometer. All experiments were performed in triplicates and the average values were determined.

2.6 Antibiotics Resistance Pattern

Kirby-Bauer disk diffusion method was used for determined the antibiotic resistance patterns of the multiple heavy metal resistance isolates towards twelve antibiotics. The antibiotics (µg/disc) from (MAST/UK) were CN: Gentamicin (10), AK: Amikacin (30), CAZ: Ceftazidime (30), CTX: Cefotaxime (30), C: Chloramphenicol (30), CIP: Ciprofloxacin (5), LEV: Levofloxacin (5), SXT: Cotrimoxazole (Sulfamethoxazole/Trimethoprim) (25), E: Erythromycin (15), TE: Tetracycline (30), VA: Vancomycin (30) and IPM: Imipenem (10) [15].

Antibiotic-impregnated discs were placed over freshly prepared Mueller Hinton agar seeded with the bacterial strains under study. All 12 antibiotic disks were placed on each of the seeded then plates were incubated at 37°C for 24 h. Zones of inhibition were obtained by measuring the diameter across the center of each zone in millimeters. The resistance breakpoints were those recommended by Clinical and Laboratory Standards Institute (CLSI) [16].

3. Results and Discussion

3.1 Isolation of Heavy Metal Resistant *Acinetobacter* from the Collected Samples

Out of 80 Diesel fuel polluted soil samples were collected from Al-Dora, Baghdad, Iraq, a total of 24 bacterial cultures identified up to genus level as *Acinetobacter* by morphological, physiological and biochemical characteristics. These isolates (coded KNZ–1 to KNZ–24) were screened for heavy metals resistance by culturing on nutrient agar medium–supplemented with 10, 25, 50 and 100mg/L of the three heavy metals; Hg^{2+}, Pb^{2+} and Cd^{2+}. Observation the growth of the obtained isolates on the nutrient agar medium revealed that 6 (25%) isolates (KNZ–3, KNZ–5, KNZ–8, KNZ–12, KNZ–16 & KNZ–21) showed good growth and selected as a multiple heavy metal resistant (MHMR) *Acinetobacter*.

The elevated concentrations of some heavy metals are found in agricultural soils located in surrounding areas of some industries which exceed the tolerable limit. Lead and Cadmium which are major contaminants found in this environment [17]. An excess of such chemicals in the water and soils is harmful to the health of the people crammed into the area [18]. Several microorganisms have developed detoxification and respiration mechanism using heavy metals and thus become resistant to it. The isolation of heavy metal resistant bacteria is significant for its metal accumulation capability along with its resistance capacity [19]. *Acinetobacter* strains play an important role in the resistance and removal of heavy metals [20]. The majority of hydrocarbon constituents of diesel fuels are biodegraded by several microorganisms commonly occurring in soil, the bioremediation of soils and sites contaminated by diesel fuels is often limited by the poor biodiversity of indigenous microflora and indigenous specialized microbes with the complementary substrate specificity required to degrade the different hydrocarbons occurring at the contaminated site [21, 22, 23].

3.2 Maximum Tolerable Concentrations (MTCs) of *Acinetobacter* on Solid Media

The isolated 24 bacterial strains were screened for the maximum tolerable concentrations (MTCs) toward increased concentrations of the previously tested heavy metals using TSM agar medium. Out of 24 isolates, 6 (25%) isolates (KNZ–3, KNZ–5, KNZ–8, KNZ–12, KNZ–16 & KNZ–21) were selected as a multiple heavy metal resistant (MHMR) organisms where it showed a high degree of resistance to all tested heavy metals with maximum tolerable concentrations (MTCs); 100–200mg/L for Hg^{2+}, 300-600mg/L for Cd^{2+} and 100–300mg/L for Pb^{2+} (Table 1).

Table 1. MTCs of the heavy metals (Hg^{2+} Cd^{2+} and Pb^{2+}) for MHMR isolates.

Isolate No.	Heavy metal concentration (mg/L)		
	Hg^{2+}	Cd^{2+}	Pb^{2+}
KNZ–3	100	350	100
KNZ–5	150	500	100
KNZ–8	150	400	150
KNZ–12	100	300	150
KNZ–16	200	600	300
KNZ–21	150	450	250

3.3 Determination of MTCs of KNZ–16 isolates in Broth Media

The selected multiple heavy metal resistant (MHMR) (KNZ–16) isolates was inoculated in TSM broth medium supplemented with the same concentrations as in solid media. This experiment was conducted by using TSM broth medium in order to minimize the complications of heavy metals and to give an accurate estimation for MTCs [24]. It was found that MHMR *Acinetobacter* KNZ–16 isolates exhibited the same resistance pattern to the tested concentrations of the different heavy metals as in solid media. The results of MTCs in broth medium are represented in Figs. 1, 2 and 3.

The results revealed that the maximum tolerable concentrations of KNZ–16 isolates were 600mg/L, 200mg/L and 300mg/L of the heavy metals Cd^{2+}, Hg^{2} and Pb^{2+} respectively.

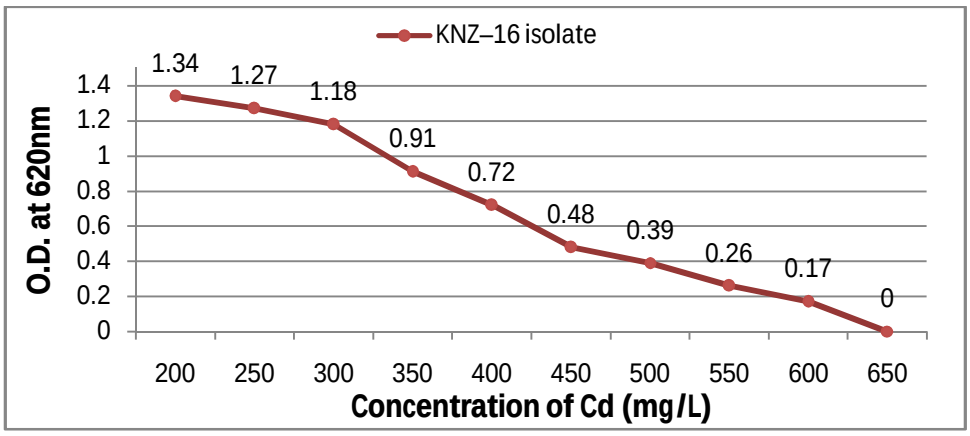

Fig. 1. MTC of MHMR KNZ-16 isolates in TSM broth medium with different concentrations of Cd (incubation at 37°C for 48 h).

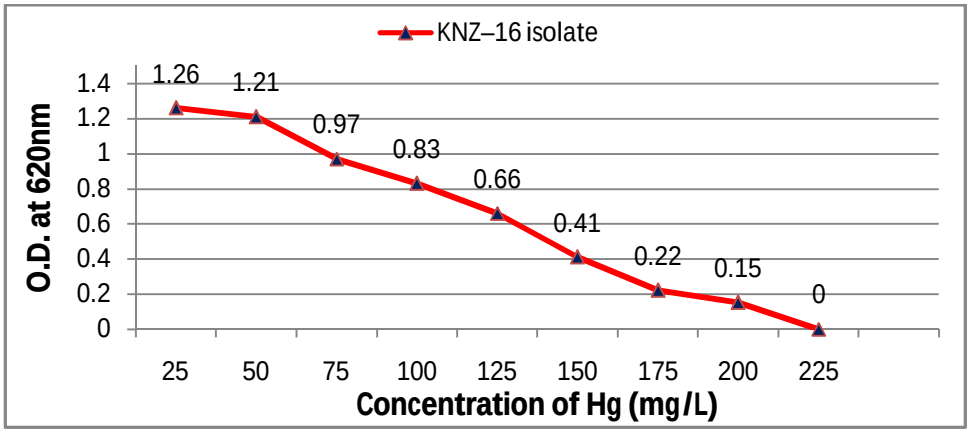

Fig. 2. MTC of MHMR KNZ-16 isolates in TSM broth medium with different concentrations of Hg (incubation at 37°C for 48 h).

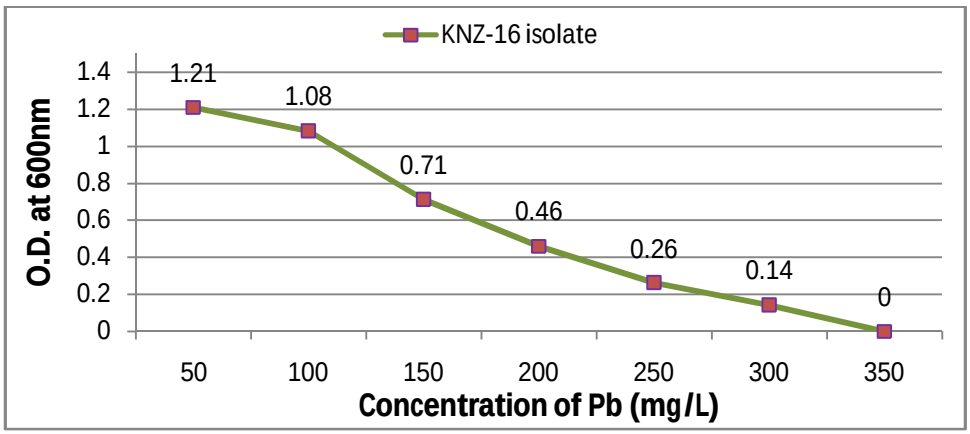

Fig. 3. MTC of MHMR KNZ-16 isolates in TSM broth medium with different concentrations of Pb (incubation at 37°C for 48 h).

Bejestani *et al*., (2013) [25] revealed that *Acinetobacter* sp. HM_AF14 was resistant to multiple metals. It also exhibited resistance to mercury and had the extensive capability of taking up zinc.

The obtained MTCs are in the range of MTCs obtained by Rohini and Jayalakshmi (2015) [26] where they discussed MTCs of *Bacillus cereus* isolated from industrial wastewater against nickel, lead, cobalt, chromium, cadmium and mercury. The strain demonstrated MTCs of 100mg/L against cobalt and cadmium, 400mg/L against chromium, 500mg/L of nickel and lead respectively making it a very potential. The microbial tolerance to heavy metals is attributed to various detoxifying mechanisms such as binding of metal in bacterial cell envelopes, complexation by exopolysaccharides, metal reduction, metal efflux or using them as terminal electron acceptors in anaerobic respiration [27]. One of the studies indicated to the high number and percentage of bacteria isolates from the petroleum polluted soils were able to tolerate seven heavy metals (Pb, Ni, Cr, Cd, Co, Cu and V) and therefore they could be useful in the bioremediation of hydrocarbon–heavy metal polluted environment [28].

3.4 Antibiotic Resistance Pattern of MHMR Isolates

The antibiotic resistance pattern of the multiple heavy metal resistant (MHMR) *Acinetobacter* isolates was studied by Kirby-Bauer disk diffusion method against 12 different antibiotics. Several reports discussed antibiotic resistance pattern in the heavy metal resistant bacterial strains isolated from soil [29, 30, 31].

Out of 6 isolates, 4 (66.6%) isolates were multiple drug resistance (MDR) where it showed high resistance degrees to the tested antibiotics. These isolates were; KNZ–5 (resistance to 8 antibiotics), KNZ–8 (resistance to 9 antibiotics), KNZ–16 (resistance to 10 antibiotics) and KNZ–21 (resistance to 9 antibiotics). While 2 isolate, KNZ–3 and KNZ–12 showed resistance to 3 and 2 antibiotics respectively. Data of antibiotic resistance pattern of MHMR *Acinetobacter* isolates are recorded in Table 2.

Table 2. Antibiotic resistance patterns of the MHMR isolates against 12 antibiotics.

Isolate No.	Tested antibiotic											
	CN	AK	CAZ	CTX	C	CIP	LEV	SXT	E	TE	VA	IPM
KNZ–3	R	S	R	S	S	S	S	S	R	S	S	S
KNZ–5	S	R	R	R	R	R	S	R	R	S	S	R
KNZ–8	R	S	R	R	R	S	R	R	R	R	S	R
KNZ–12	S	S	S	R	S	S	S	R	S	S	S	S
KNZ–16	R	R	R	R	R	R	S	R	R	R	R	S
KNZ–21	R	S	R	R	R	S	R	R	R	R	R	S

CN: Gentamicin, AK: Amikacin, CAZ: Ceftazidime, CTX: Cefotaxime, C: Chloramphenicol, CIP: Ciprofloxacin, LEV: Levofloxacin, SXT: Cotrimoxazole (Sulfamethoxazole/Trimethoprim), E: Erythromycin, TE: Tetracycline, VA: Vancomycin, and IPM: Imipenem.

The isolates (KNZ–5, KNZ–8, KNZ–16 & KNZ–21) were selected as MAR isolates. Among them, KNZ–16 isolate was the highest antibiotic resistant isolate where it showed a wide range of resistance to all tested antibiotics; Gentamicin, Amikacin, Ceftazidime, Cefotaxime, Chloramphenicol, Ciprofloxacin, Cotrimoxazole (Sulfamethoxazole/Trimethoprim), Erythromycin, Tetracycline, Vancomycin except Levofloxacin and Imipenem. Microorganisms resistant to antibiotics and metals appear as the result of exposure to metal-contaminated environments which cause coincidental coselection of resistance factors for antibiotics and heavy metals. Heavy metal tolerance in the environment may contribute to the maintenance of antibiotic resistance genes by increasing the selective pressure of the environment [32].

Resistance genes that afford metal resistance often fall within the class of resistance mechanisms called "efflux pumps". Notably, efflux pumps also provide resistance to a wide range of antibiotics and biocides [33, 34]. The combined expressions of antibiotic resistance and metal tolerance may be results of selection by metals present in an environment. It was demonstrated that even at low concentrations of metals such as aluminium, copper, manganese and lead in residential soils, antibiotic resistance was selected, as evidenced by increased relative gene abundances [35]. In the study by Oyetibo and colleagues (2010) [36], five heavy metal resistant bacteria strains; *Micrococcus* sp., *Nocardia* sp., *Acinetobacter junii*, *Actinomyces turicensis* and *P. aeruginosa*, resisted all the 18 antibiotics tested. The high levels of antibiotic resistance among MHMR *Acinetobacter* isolate confirm the correlation between antibiotic and metal resistance in nature. Yamina *et al.*, (2012) [37] reported that, 13 heavy metal resistant bacteria were resistant to chromium, zinc, lead, and cadmium with minimum inhibitory concentration (MIC) ranged from 0.1 to 1.5mg/l, these isolates showed co-resistance to other heavy metals and antibiotics, of which 85% were multidrug resistant. The correlation between metal tolerance and antibiotic resistance in bacteria were established by many reports and this may be due to the presence of resistance genes to both antibiotics and heavy metals on the same plasmid in bacteria and are thus more likely to be transferred together in the environment [38, 39].

3. Conclusion

The soil contaminated with diesel fuel or others petrochemicals may be as one of the sources of dangerous bacteria which have the mechanisms to resist heavy metals and antibiotics, The public health implication and hazard derivable from these findings stem from the horizontal transfer of the genetic elements responsible for these resistance abilities to pathogenic bacteria that they might come in contact with. On the other hand, the bacteria with antibiotic and heavy metal resistance isolated and identified in this study have potential application in bioremediation of environments polluted with heavy metals and may also help to overcome the inhibition that heavy metals exert on the biodegradation of organic pollutants. Also, such dual resistant bacteria would have the capacity to compete well with antibiotic-producing flora in the polluted environment.

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