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ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF HEAVY METAL TOLERANT BACTERIA FROM MULA RIVER, PUNE, MAHARASHTRA, INDIA

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ABSTRACT
The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. The present study deals with isolation, identification and characterization of heavy metal tolerant bacteria. Heavy metal tolerating bacteria was isolated from Mula river, Chinchwad, Pune, Maharashtra, India. On the basis of morphological, biochemical, 16S rDNA gene sequencingand phylogeny analysis revealed that, the isolate MR4 was identified as *Klebsiella pneumoniae*. The *K. pneumoniae* showed optimum growth at 28°C and pH 7.0. The *K. pneumoniae* was resistant to cobalt (Co), nickel (Ni), arsenic (As), mercury (Hg) and copper (Cu). The multiple metal tolerance of the isolate was also associated with resistance to antibiotics such as ampicillin and vancomycin. Morphological changes of the *K. pneumoniae*in the presence of various heavy metals were observed by phase contrast microscopy. The identified heavy metal tolerant bacteria could be useful for the bioremediation of heavy metal contaminated sites.

KEY WORDS: heavy metal, *Klebsiella pneumoniae*, tolerance, Mula river, India

Abbreviations: MIC; Minimum inhibitory concentration, SIC; sub inhibitory concentration

INTRODUCTION

Heavy metals are stable and persistent environmental contaminants since they cannot be degraded or destroyed unlike organic pollutants and subsequently bioaccumulate in the food chain (Verma *et al*., 2001). Some heavy metals at low concentrations have physiological role in molecular metabolism, some of which are co-factors of enzymes involved in the key cellular processes, whereas others are needed for regulation of osmotic pressure, to stabilize molecules through electrostatic interactions. On the other hand some are nonessential and highly toxic (Bruins *et al*., 2000). At high concentrations, even very important heavy metal ions form unspecific complex compounds, leading to toxic effects. Natural sources contribute to heavy metal pollution to a less extent; whereas artificial sources resulted from different human activities, contribute decisively to the pollution of the environment. To date environmental pollution has become the major concern especially in developing countries like India. In spite of Government regulations, lot of such waste is dumped around the cities and metropolis and deposited in natural water resources. In Pune, Mula river is contaminated with such waste materials (Imandoust *et al*., 2007). Khatat *et al.* (2003) reported that elevated levels of toxic component in river water, indicated by reduced fish population in the river. Therefore, we isolated bacteria from this river and further heavy metal tolerance was studied.

The introduction of heavy metals in various forms in the environment can results inconsiderable modifications of the microbial communities and their activities (Sheik *et al*., 2012).Although heavy metals are toxic to humans as well as microbes, metals and microorganisms have co-existed since early history (Silver and Phung, 1996) and their survival in polluted environment depends on intrinsic biochemical and structural properties, physiological, or genetic adaptation including morphological changes of cells, as well as environmental modifications of metal speciation (Ehrlich, 1997; Wuertz and Mergeay, 1997). Generally, the strategy adopted by microorganisms aims to avoid the buildup of excess metal levels, and thus to prevent the onset of toxicity symptoms. These heavy metal resistant microbes develop the various mechanisms which helpin detoxification and removal of the heavy metal from polluted environment (Ahmed *et al*., 2005). This possible environmental application to remove dangerous heavy metal from the contaminated sites generated present interest to screen out themetal resistant microbes from the contaminated site and its characterization to explore it further for its suitability for bioremediation of heavy metal contaminated environment.

MATERIALS AND METHODS

Isolation of heavy metal tolerant bacteria

For isolation of the heavy metal tolerant bacteria, water samples of various origins were collected from river Mula, Pune, Maharashtra, India, in a 500 ml sterile stoppered bottle. Samples were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}& 10^{-5}) in sterile saline (0.85 % w/v NaCl) and 100 µl of each dilution was spread plated on nutrient agar (NA) plates, each containing 0.1 mM concentrations of heavy metals such as Sodium arsenate ($Na_2HAsO_4.7H_2O$), Sodium arsenite ($NaAsO_2$), Copper sulfate ($CuSO_4$), Nickel chloride ($NiCl_2$), Cobalt chloride ($CoCl_2$) and Mercury chloride ($HgCl_2$). The plates were incubated at 28°C for 48 hrs to 72 hrs (till colonies developed). Colonies with distinct morphology were selected and maintained as glycerol stock (25% v/v).

Morphologically distinct and independent fifteen isolates were picked from plate and further tested in Luria Bertani broth (LB) with 0.5 mM concentration of above mentioned heavy metals separately in 24 well microtiter plates and growth was measured in terms of optical density (OD) at 600nm. Among them, six isolates which showed good growth in presence of 0.5 mM heavy metals were selected and determined the MIC of all six isolates. For this purpose, 1% overnight growth of each isolate was added to wells containing different concentration of heavy metals (1-64 mM for salt of cobalt, nickel, copper; 10-500 mM for salt of arsenate; 0.5- 10mM for salt of arsenite; 0.2-3.2 mM for salt of mercury) and incubated at 28°C for 24 hrs. Growth of isolates was measured in terms of optical density (OD) at 600nm. The bacterial isolate which showed highest MIC for heavy metals was selected for further study.

Identification of isolate

The bacterial isolate (MR4) which showed highest MIC for tested heavy metals was identified by sequencing the 16S rRNA gene fragment. The DNA from the isolate MR4 was extracted by using Uniflex DNA extraction kit (Bangalore Genei, India) as per manufacturer's instructions. The 16S rDNA gene was amplified by PCR using bacterial universal primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-TAC GGC TAC CTT GTT ACG ACT T-3′). The PCR was performed with a cycle of 94°C for 4 min; then 40 cycles of 94°C for 1 min, 55°C for 45s, and 72°C for 1 min and 30s each; with final extension of 72°C for 10 min, and the mixture was held at 4°C. The amplified PCR product was checked by electrophoresis and sequenced. To identify bacteria, the 16S rDNA sequence similarity was searched by using the National Center for Biotechnology Information blast (NCBI-Blast2)-Nucleotide Database Query program. The almost entire 16S rDNA sequence of isolate MR4 was deposited in GenBank and accession number obtained.

Construction of phylogenetic tree

Phylogenetic analysis of 16S rDNA sequences were performed using MEGA version 5 software (Tamura *et al*. 2011). The nucleotide sequences were compared with the sequences available at NCBI GenBank database. Neighbour-joining tree method was used for the construction of phylogenetic tree **(Saitou and Nei, 1987)**.

Biochemical characteristics

Biochemical characteristics of the isolate MR4 were studied by using standard methods. The tests included Gram nature, motility, utilization of different carbon sources, catalase and oxidase test.

Optimization of growth parameters

pH

Optimal pH was determined so that this optimum parameter could be used in subsequent experiments. The isolate was grown in LB medium with varying pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 7.6, 8.0, 9.0, and 10.0 and incubated at 28°C for 24 hrs at 180 rpm. Microbial growth of each sample was determined in terms of optical density at 600 nm using UV- Vis spectrophotometer.

Temperature

Optimal temperature was determined so that this optimum parameter could be used in subsequent experiments. The isolate was grown in LB medium and incubated at temperature ranging from 15°C, 20°C, 28°C, 37°C, 45°C, and 55°C for 24 hrs at 180 rpm. Microbial growth of each sample was determined in terms of optical density at 600 nm.

Determination of antibiotic resistance

Since most of the metal tolerant bacteria are also antibiotic resistant, antibiotics sensitivity was performed by using the Kirby-Bauer Disk Diffusion Test (KBDD). KBDD was performed by spreading 100 µl culture on each LB plate and then antibiotic discs (Hi-media, India) were placed onto the agar surface and the plates were incubated at 28°C for 24

hrs and observed for zone of inhibition around the disk. The sensitivity and resistance profile was determined based on the diameter of the zone of inhibition.

Determination of minimum inhibitory concentration

The minimum concentration of the metal inhibiting complete growth of microorganism i.e. MIC, was determined by broth microdilution method. Six different heavy metals viz., $Na_2HAsO_4.7H_2O$, $NaAsO_2$, $NiCl_2$, $CoCl_2$, $CuSO_4$ and $HgCl_2$ were used for MIC determination. Stock solution of each heavy metal was prepared in distilled water and then filter sterilized under aseptic conditions. Tubes for different concentration of above mentioned heavy metals (1-32 mM for salt of cobalt, nickel, copper; 50-400 mM for salt of arsenate; 0.2-3.2 mM for salt of mercury and 1 to 6mM for salt of arsenite) were inoculated with appropriate cell suspension grown in LB without heavy metal to obtain initial optical density of approximately 0.05. Growth of bacterium was monitored by measuring the optical density (OD) at 600nm after 24 h incubation at 28°C.

Effect of heavy metals on cellular morphology

The effect of heavy metals on cellular morphology was observed under phase contrast microscope. For this purpose, 1% inoculum of isolate MR4 was inoculated into LB broth containing sub inhibitory concentration (SIC) of one of the following heavy metals: Ni(II) (8mM), Co(II) (8mM), Cu(II) (16mM), As(III) (2.5mM), Hg(II) (0.8mM) and As(V) (200mM) and without heavy metal (control). Then tubes were incubated at 28°C in incubator shaker (180 rpm) for 24 hrs. Grown culture was spotted onto clean and grease free slide and viewed by phase contrast microscopy and images were captured by Image pro™ discovery software (Olympus).

RESULTS AND DISCUSSION

Isolation and identification of heavy metal tolerant bacteria

In the present study, 130 bacterial colonies were randomly selected from nutrient agar plates containing various heavy metal salts. Out of them, morphologically distinct and independent fifteen isolates were assessed for their growth performance in 0.5 mM of different heavy metals. As can be seen in Fig. 1 six isolates (isolate no. 1, 4, 6, 15, 21 and 32) showed good growth in presence of all metals.

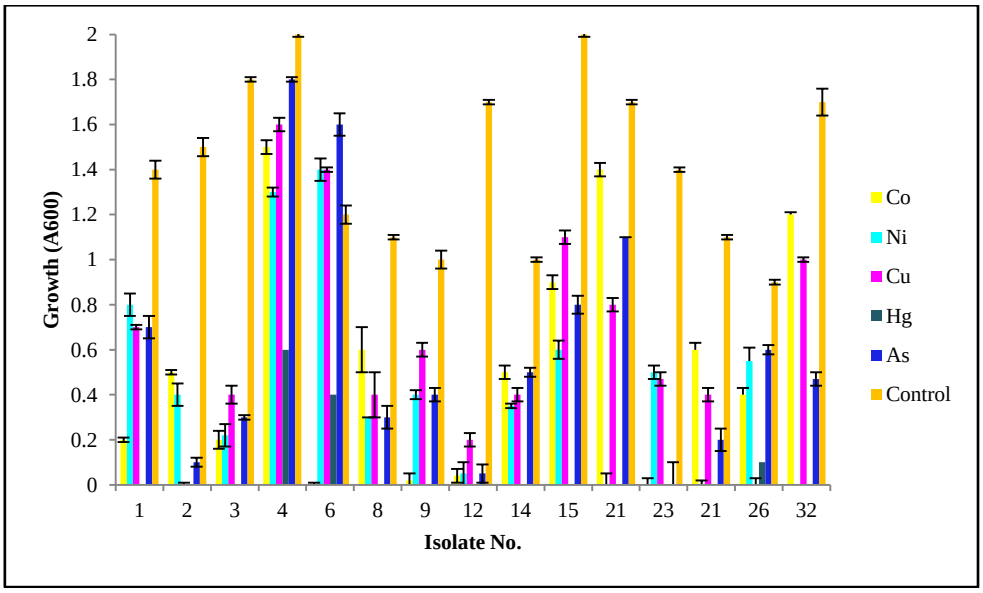

Figure 1 The growthcharacterization of fifteen isolates in presence of 0.5 mM heavy metals

Further these six isolates were selected for screening of heavy metal tolerant bacteria. Among them, the isolate MR4 exhibited maximal tolerance to multiple heavy metals i.e. As(V), As(III), Cu(II), Co(II), Ni(II), and Hg(II), as seen by monitoring growth at 600nm and was thus chosen for further studies (Table 1).

Table 1 MIC of heavy metals studied for six bacterial isolates

Bacterial Isolate	MIC* (mM) of				
	Arsenate	**Copper**	**Cobalt**	**Nickel**	**Mercury**
1	80	8	4	8	-
4	**>400**	**32**	**16**	**16**	**1.6**
6	60	4	-	4	-
15	100	4	4	4	-
21	200	2	2	-	-
32	**100**	**2**	**2**	-	-

***** Determined on LB supplemented with varied concentration of heavy metalsat 28°C in 24hrs

The almost complete 16S rDNA nucleotide sequence of isolate MR4 is given in fig. 2. It was identified as *Klebsiella pneumoniae* and its sequence was submitted in GenBank as *K. pneumoniae* strain VD and its gene accession number is **HQ857583.1**.

The fig. 3 represents the phylogenetic dendrogram showing the relationship of isolates MR4 with the most closely related strains.

>*K. pneumoniae* strain VD

TCCAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGC
ACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAG
GGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGG
CCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGAT
CCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTT
CGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTACCCG
CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAA
TTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGA
ACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATG
CGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTG
TGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTA
AAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAA
GAACCTTACCTGGTCTTGACATCCACAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTGTGAGAC
AGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTG
GGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATACAAAG
AGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTC
GACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCC

Figure 2 Complete sequence of *Klebsiella pneumoniae* stain VD

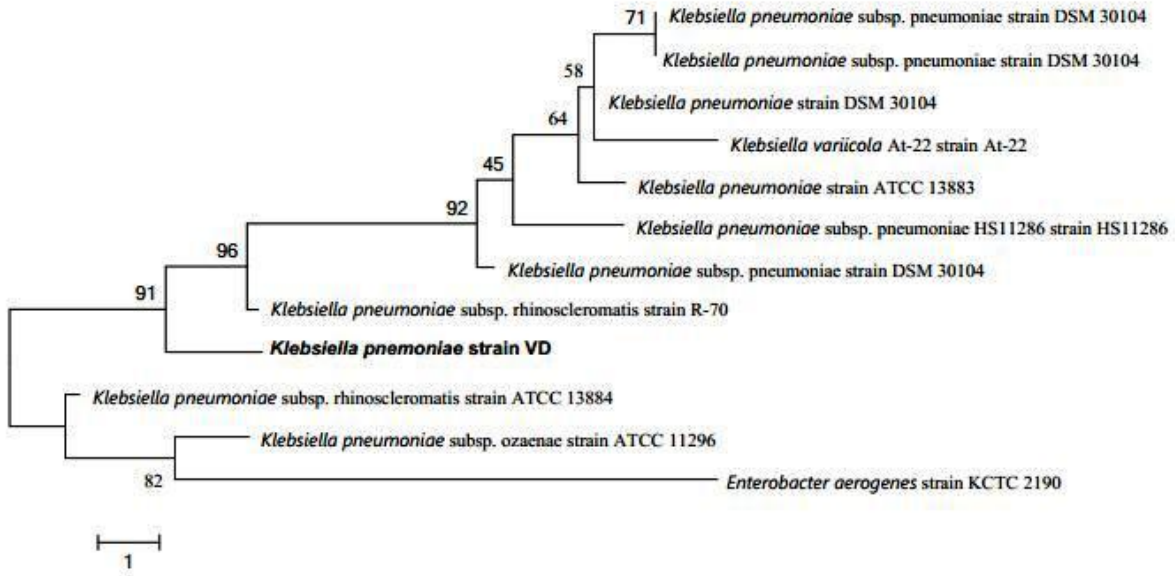

Figure 3. Phylogenetic dendrogram based on 16S rDNA gene sequence showing the relationship of isolate MR4 with the most closely related strains.

Biochemical characterization

The isolate MR4 was studied for their biochemical characteristics. The isolate MR4 is gram-negative, non-motile, rod-shaped bacterium showed positive catalase test and oxidase negative. Carbohydrate utilization studies have shown that the isolate MR4 was positive for lactose, fructose, galactose, glucose, maltose, dextrose, sucrose and some other sugars mentioned in Table 2 and change in color as a result was compared with standard chart provided along with the kit.

Table 2 Carbohydrate utilization profile of isolate MR4

Sugar	Result
Lactose	+
Xylose	+
Maltose	+
Fructose	+
Dextrose	+
Galactose	+
Raffinose	+
Trehalose	+
Melibiose	+
Sucrose	+
L-Arabinose	+
Mannose	+
Insulin	-
Sodium Gluconate	-
Glycerol	+
Salicin	-
Dulcitol	-
Inositol	+

+: Positive test; -: Negative test

Optimization of growth parameters

The isolate MR4observed to be capable of growing within a broad pH range, from 4-9 and within broad temperature range (20°C to 45°C). In this study the *K. pneumoniae* exhibited a broad pH and temperature range suggesting the adaptability of *K. pneumoniae* to varied environmental conditions and optimum pH and temperature for its growth were 7 and 28°C respectively [Fig 4 (A and B)].

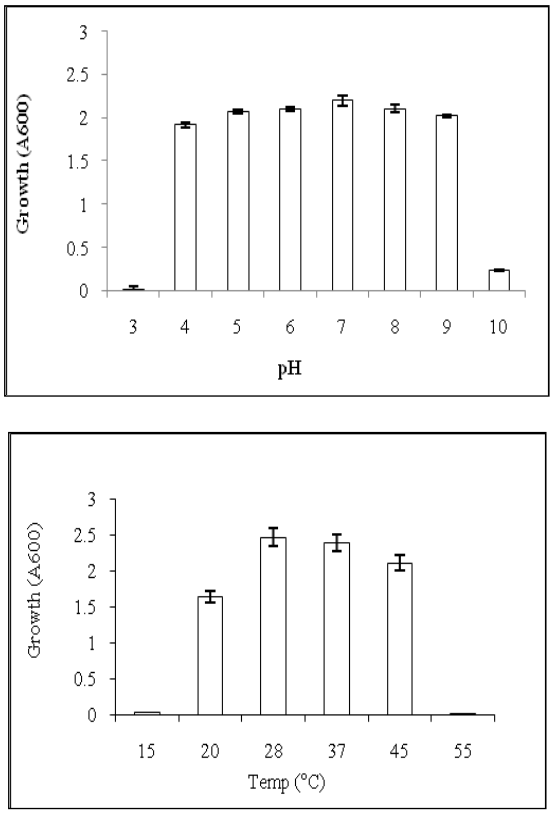

Figure 4 Growth of isolate MR4 at different pH (**A**) and at different temperature (**B**)

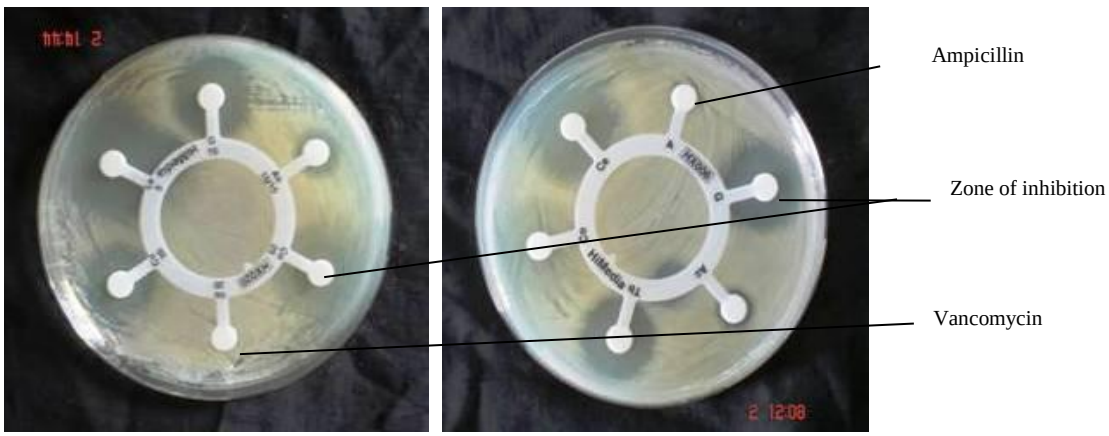

Figure 5 Antibiotic resistance and sensitivity of *K. pneumoniae*

Antibiotics and heavy metal resistance of *K. pneumoniae*

The MIC of various heavy metals that can completely inhibit the growth of *K. pneumoniae* was determined. The MIC of various heavy metals for *K. pneumoniae* was given in fig. 6. In most of the studies, metal resistance has been reported to hold an association with antibiotic resistance (Verma *et al*., 2001), so *K. pneumoniae* was tested for its antibiotic resistance and susceptible profile. As shown in Table 3. *K. pneumoniae* strain VD is resistant to ampicillin (10µg) and vancomycin (30µg) and sensitive to most of the antibiotics tested (Fig. 5). A correlationexists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that resistance genes to both (antibiotics and heavy metals) may be located closely on the same plasmid in bacteria and are thus more likely to be transferred together in the environment. Underconditions of metal stress, metal and antibioticresistance in microorganisms possibly helpsthem to adopt faster by the spread of resistantfactors than by mutation and natural selection(Silver and Misra 1988).

Table 3 Antibiotic resistance and sensitivity of *K. pneumoniae*

Antibiotic	**Symbol**	**Antibiotic/disc (µg)**	**Inhibition zone diameter (cm)**
Cephatoxime	Ce	30	2.4
Levofloxacin	Le	5	2.1
Aztreonam	Ao	30	1.675
Amikacin	Ak	30	1.8
Imipenem	I	10	1.6
Ceftazidime	Ca	30	1.91
Tetracycline	T	30	1.75
Gentamicin	G	10	1.8
Co-Trimoxazole	Co	25	1.64
Cefuroxime	Cu	30	1.8
Ofloxacin	Of	5	2.16
Ampicillin	A	10	Resistant
Tobramycin	Tb	10	1.65
Vancomycin	Va	30	Resistant
Ampicillin/Sulbactam	As	10\10	1.24
Streptomycin	S	10	1.7
Nalidixic Acid	Na	30	2.1
Ciprofloxacin	Cf	5	2.8
Doxycycline	Da	30	1.7
Chloraphenicol	C	30	2.4
Cephalexin	Cp	30	1.65
Oxytetracycline	Of	30	2.25
Furazolidone	Fr	50	1.2
Augmentin	Au	30	1.35
Kanamycin	K	30	1.85
Ticarcillin	Ti	75	1.8
Sparfloxacin	Sc	5	2.2
Maxifloxacin	Mo	5	2
Gatifloxacin	Gf	5	2
Ceftriaxone	Ci	30	2.4
Cefpodoxime	Cep	10	1.65
Furazolidone	Fr	50	1.2

Legend: * Antibiotics sensitivity was performed by spreading 100 µl culture on each LB plate and then antibiotic discs (Hi-media, India) were placed onto the agar surface and the plates were incubated at 28°C for 24 hrs and observed for zone of inhibition around the disk.

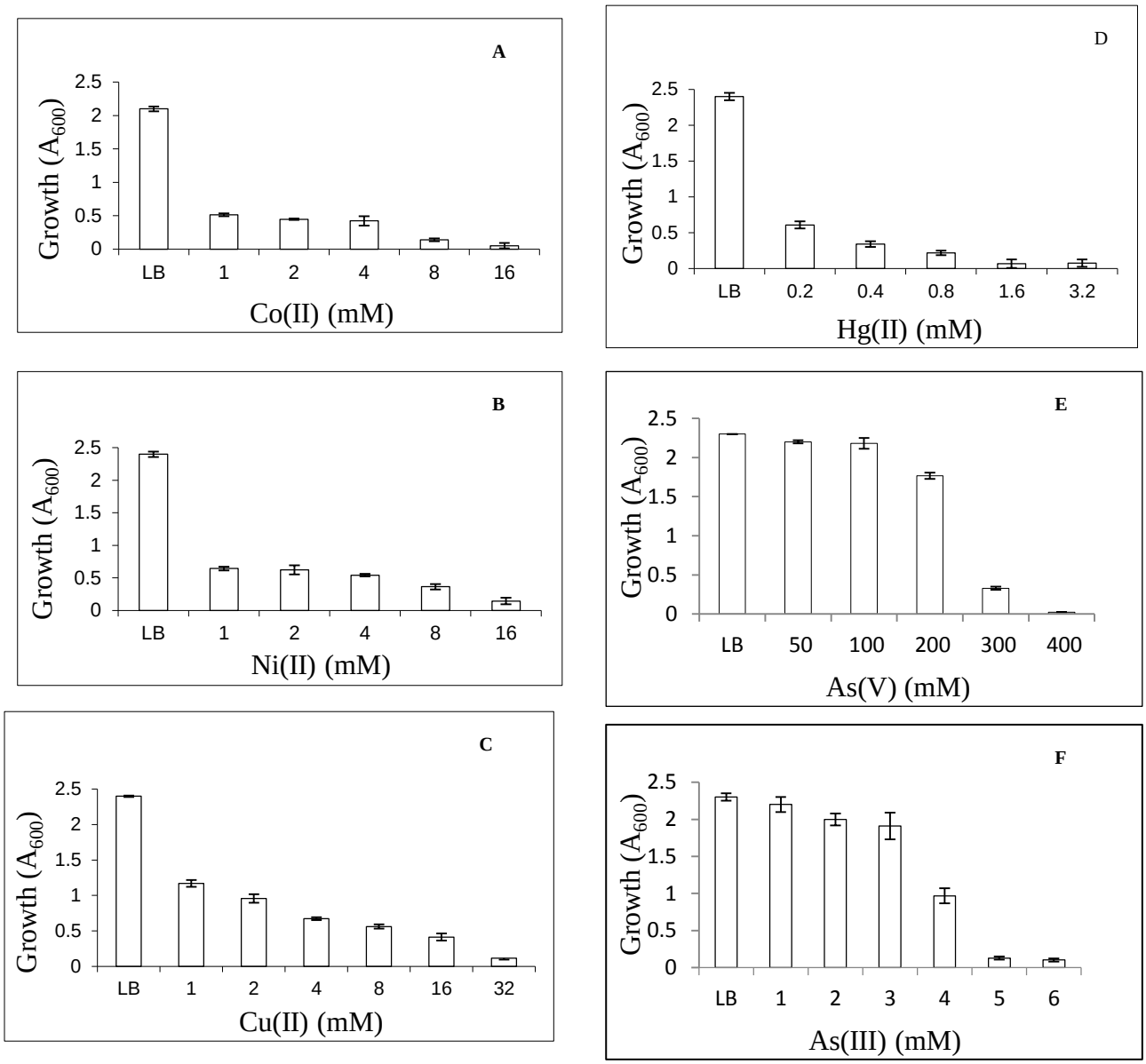

Figure 6 Minimum inhibitory concentration of different heavy metals for *K. pneumoniae*.

Morphological changes of *K. pneumoniae* in presence of heavy metals

Morphological changes of *K. pneumoniae* under various heavy metals were studied by phase contrast microscopy. The morphology of *K. pneumoniae* showed significant changes in presence of various heavy metals. Cellular morphology was unchanged after growth in LB broth containing sub inhibitory concentration of As(V) and As(III) whereas cell became elongated in Cu(II), Hg(II) and Co(II) and cells became rounded in presence of Ni(II) (Fig.7). This suggests that arsenate and arsenite are not toxic to the isolate under the condition tested. In Cu(II), Hg(II) and Co(II) reduced bacterial growth and cell became elongated indicating possible interference of these metals in the regulation of bacterial cell wall biosynthesis (Vaituzis *et al*., 1975). Cell elongation could result from incomplete reproduction process because of the starvation related lack of structural molecules (Fulladosa *et al*., 2006).

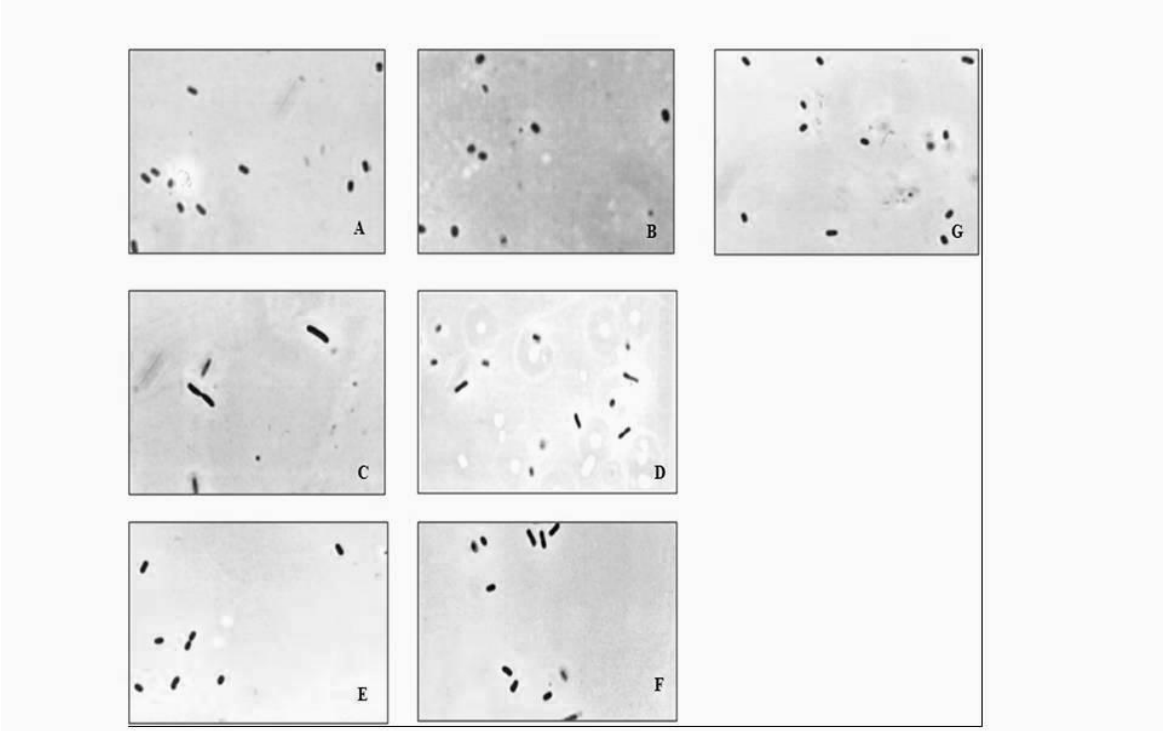

Figure 7 Phase contrast microscopic observations on *Klebsiella pnuemoniae* (1000 X) grown at sub inhibitory concentration (SIC) of following heavy metals A] LB without metal; LB with B] 8mM Ni(II) C] 8mM Co(II) D] 16mM Cu(II) E] 2.5mM As(III) F] 0.8mM Hg(II) G] 200mM As(V).

CONCLUSION

The overall study shows that out of these 15 isolates from Mula river, the isolate MR4 (*K. pneumoniae*) have high capacity to tolerate multiple heavy metals. This isolate could help in the effective bioremediation of heavy metal contaminated sites.

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