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Antibiotic resistance in heavy metal resistant non-lactose fermenting *Enterobacteriaceae* colonies cultured from textile industry effluents

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ABSTRACT

Many of the aquatic pathogenic microflora belong to the non-lactose fermenting (NLF) *Enterobacteriaceae* and emergence as well as the spread of antibiotic resistance in them is now a serious threat to public health worldwide. Growth of textile industries has heavily flourished acting as hub for the ready-made garments factories in Bangladesh. Textile industry effluents may contain heavy metals and it is not clear whether their disposal is strictly monitored or not and their impact on the aquatic microflora have not been properly investigated and monitored. To assess the impact of heavy metals on the emergence and spread of antibiotic resistance in NLF *Enterobacteriaceae*, samples were collected from four different locations of drains running down effluents from five (n=5) different textile industries located in Savar, Dhaka, Bangladesh. Total bacterial counts (TBCs), minimal inhibitory concentrations (MICs) of heavy metals, antibiotic susceptibility, binary exposure and plasmid profile experiments were performed. TBCs from day 1 to day 5 revealed that most colony forming units (CFUs) uncountable at 10^{-6} dilution could be counted at 10^{-10} dilution. A total of 100 NLF bacterial isolates were categorized as type-1 and type-2 using 4 differential media. The MICs of 100% isolates in both type-1 and type-2 for nickel (Ni), lead (Pb) and chromium (Cr) were 0.6 mM, 0.6 mM and 1.0 mM, respectively. In case of type-1, most (38%) showed resistance to azithromycin (AZM) and least (2%) to cefotaxime (CTX). In case of type-2, most (48%) showed resistance to AZM and least (8%) to CTX. Binary exposure experiments revealed the combined effect of heavy metals and antibiotics. Where in most cases, zone of inhibition increased or remained unchanged. In few cases, zone of inhibition decreased. This finding indicated that the heavy metals typically exert negative or no effects on antibiotic resistance in the isolates tested. Plasmid profiling of the type-1 and type-2 NLF isolates resistant to both the antibiotics and heavy metals revealed that one of the five type-1 isolates contained very large plasmid (14 Kb) and four of the eight type-2 isolates contained 13 Kb plasmids and two contained 12 Kb plasmids. These findings indicated that these isolates possibly possess the ability to sequester metals before being discharged into the soil and/or water environment.

Keywords: Antibiotics, effluents, heavy metals, minimum inhibitory concentration (MIC), resistance.

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INTRODUCTION

Environmental problems caused by heavy metal pollution are global because these metals cannot be broken down and, hence, they are potentially toxic (Zhao et al., 2016; Fashola et al., 2016). Heavy metals include zinc, copper, nickel, lead, chromium, cadmium etc. are usually released by various industries, such as the textile and chromate manufacturing, wood preservation, leather tanning and dye industries considering as long-term sinks

for such elements (Igiri et al., 2018; Ferraz et al., 2012; Cabrera et al., 2007; Unaldi et al., 2004). Typically, the effluents discharged by industrial plants into drains leading to the river are used for irrigation and other domestic uses. By interacting with the polluted water, the flora and fauna of the river system sequester the potentially toxic elements slowly over a long period of time. Increased use of river water for daily purposes could have long-term implications for water quality including water microorganisms and the transfer of zootoxic elements into human diets (Medfu Tarekegn et al., 2020; Coral et al., 2005). During the microbiological process, microorganisms use chemical contaminants as an energy source, and reducing the pollutants from the environment is one kind of bioremediation process. However, excessive amounts of inorganic nutrients in the environment cause microbial inhibition (Ahirwar et al., 2016).

By developing resistance mechanisms, microbes are able to tolerate metal toxicity and later on can be used for bioremediation. As such, antibiotic and metal ion resistant microbes are potentially health hazardous though used for bioremediation and usually appear as a consequence of exposure to metal contaminated environments and as a result of co-selection and formation of antibiotic and metal resistance transmissible plasmids (Abd Elhady et al., 2020; Khan, 2016). These bacteria are therefore a double-edged sword. Usually, these microbes are contained within plasmid DNA; however, they can also be coupled to chromosomal DNA (Jain et al., 2009; Vajiheh et al., 2003; Zou Algharneyn et al., 2007). They can be used in a heavy metal contaminated site in a more efficient way than available chemical remediation methods for the overall metal content reduction (Smith et al., 2015; Azubuike et al., 2016).

Many of the aquatic pathogenic microflora belong to the NLF *Enterobacteriaceae*. Emergence of antibiotic resistance in them and their spread are now a serious threat to public health worldwide. Earlier, Ashikuzzaman et al. reported heavy metal resistance in gram positive bacteria isolated from textile industry effluents; Fakruddin et al. (2009) reported chromium resistance bacteria; Hossain and Anwar (2012) reported chromium and copper resistant bacteria isolated from tannery effluents. Growth of textile, ready-made garments, dyeing and tannery industries have heavily flourished in Bangladesh. However, it is not certain whether effluent treatment plants (ETPs) have been installed or not. Even if installed, it is not certain whether those are functional or not. Furthermore, scopes of researchers to monitor the effluents before being discharged into soil or aquatic environment are limited. Textile industry effluents may contain heavy metals and their impact on the aquatic microflora have not been properly investigated and monitored in Bangladesh textile industry areas. Therefore, the present study sought to assess the impact of heavy metals on the emergence and spread of antibiotic resistance in NLF *Enterobacteriaceae*.

MATERIALS AND METHODS

Study area and collection of sample

Samples were collected in sterilized airtight 1.5 L glass bottle (Pyrex, UK) from five (n=5) different drains running in Savar Upazila industrial area during a three months period from June to August 2016. The effluents were discharged directly from different textile industries into the drains and the drains were connected to the nearby Bangsi and Turag rivers. A total of five samples were collected from five different locations with an average distance of 2.0 m from each drain going downhill. The collected samples were brought to the laboratory, maintained at 4°C for further studies and investigated within two hours of collection. Names of the industrial drains, dates of collection, sites of collection along with longitude and latitude are mentioned in Table 1. The bacteriological study was carried out in the Common Research Laboratory as well as in the Research Laboratory for Biomedical Sciences, Department of Biochemistry and Molecular Biology, Jahangirnagar University, Savar, Dhaka.

Total bacterial colony (TBC) count

The textile effluent samples were serially diluted with autoclaved distilled water starting at 10^{-1} and ending up at 10^{-10} times and 50.0 µl aliquot (2.5 µl/ml) of the 10^{-6} to 10^{-10} serially diluted effluents of each sample were inoculated by spreading them in petri-plates containing 20.0 ml plate counting agar (PCA) and incubated at 37°C for 5 consecutive days to observe the appearances of bacterial colony and count the total bacterial colony **(**TBC). The appeared colony forming units per milliliter (CFUs/ml) were counted manually with naked eyes and under light microscope (XSZ-107 BN, 230v, USA).

Selection of bacterial colonies using differential media

To enrich the number of aerobic bacteria, 1.0 ml aliquot of the collected effluent from each location was inoculated in 1.0 ml of autoclaved nutrient broth (NB) in 10.0 ml test-tube and incubated at 37°C for 24 h in an orbital shaker incubator at a shaking speed of 180 rpm. After 24 h, the culture was inoculated in petri-plates containing differential media using cotton swab and by streaking. Differential agar media, MacConkey (MAC), Xylose-Lysine-Deoxycholate (XLD), *Shigella-Salmonella* (SS) and Thiosulphate Citrate Bile Salt Sucrose (TCBS) were used for the identification of the gram negative NLF *Enterobacteriaceae* colonies (Bacteriological Analytical Manual, 1998). Bacterial colonies that appeared on differential agar petri-plates were picked up and purified by repeated plate streaking method (Cappuccino and Welsh, 2017). The purity of each bacterial colony morphology of the selected NLF *Enterobacteriaceae* isolates was ensured by observing their color, size and shape by comparing with those of the standard colonies of *Shigella flexneri* 2a (UniProt accession number: AE005674), and *Salmonella* Serovar paratyphi B (UniProt accession number: CP000886) under the microscope. These standard colonies that grew on the four differential media were used as positive control. The selected isolates were grown in nutrient broth supplemented with 0.3% yeast extract and stored at -80°C after the addition of 15% glycerol.

Determination of minimum inhibitory concentrations (MICs) to heavy metals

The petri-plates were spot inoculated using plate assay method by drawing square patches on them and incubated at 37°C for 24 h to

Table 1. Specific locations of sample collection points in drains carrying textile industry effluent.

Sample	Date of collection	Location distance	Distance Meter (m)	Industries	Longitude	Latitude
S1	June 26, 2016	L1----L2	2.0 m	Pakiza Textiles Ltd.	90°15'29"E	23°50'32"N
		L2----L3	1.5 m			
		L3----L4	2.5 m			
		L4----L5	2.0 m			
S2	August 1, 2016	L1----L2	1.0 m	Aman Spinning Mills	90°19'9"E	23°54'23"N
		L2----L3	2.0 m	Mondal Fashions Ltd.	90°19'20"E	23°54'20"N
		L3----L4	1.5 m	Green Life Knit Composite Ltd.	90°19'13"E	23°54'10"N
		L4----L5	3.0 m	Eva Garments	90°19'25"E	23°53'59"N
S3	August 7, 2016	L1----L2	2.5 m	Marma Composites	90°19'9"E	23°54'2"N
		L2----L3	2.0 m			
		L3----L4	1.5 m	Fashion Garments Ltd.	90°18'50"E	23°54'48"N
		L4----L5	2.0 m			
S4	August 21, 2016	L1----L2	1.0 m	Ultra Embroidary Ltd.	90°16'31"E	23°56'12"N
		L2----L3	3.0 m			
		L3----L4	1.5 m	Amigo Fashion Ltd.	90°16'30"E	23°56'6"N
		L4----L5	2.5 m	Shine Embroidary Ltd.	90°16'52"E	23°56'9"N
S5	August 28, 2016	L1----L2	1.0 m	Dekko Designs Ltd.	90°18'16"E	23°55'36"N
		L2----L3	3.0 m	Magpie Knit Wear	90°17'22"E	23°54'35"N
		L3----L4	1.5 m	Hameem Group	90°18'22"E	23°55'50"N
				AJ Super Garments Ltd.	90°18'36"E	23°55'22" N
		L4----L5	2.5 m	Sharmin Group	90°18'24"E	23°55'55"N
				Trouser Line Ltd.	90°18'38"E	23°55'26"N

NB: S1:Sample 1; S2:Sample 2; S3:Sample 3; S4:Sample 4; S5:Sample 5; L1:Location 1; L2:Location 2; L3:Location 3; L4:Location 4; L5:Location 5.

determine the approximate minimum inhibitory concentrations (MICs) of the gram negative NLF *Enterobacteriaceae* colony isolates to three metal salts, $K_2Cr_2O_7$ (chromium salt), $NiSO_4$ (nickel salt) and $PbNO_3$ (lead salt) (Zhou et al., 2015). Briefly, the experimental petri-plates were prepared by supplementing MAC agar medium with three metal salts, $K_2Cr_2O_7$, $NiSO_4$ and $PbNO_3$ to give final concentration of 0.1, 0.3, 0.5, 1.0, and 2.0 mM for Cr^{6+}, 0.2, 0.3, 0.6, 1.25, 2.5 mM for Ni^{2+} and 0.1, 0.15, 0.3, 0.6, 1.2 mM for Pb^{2+}, respectively. On each plate, a total of 40 square patches were drawn using a marker pen. Then each patch of the plate was inoculated with a single colony isolate that was previously cultured on MAC agar plates without any metal salts. *Escherichia coli* (*E. coli*) ATCC (25922) (UniProt accession number: CP009072) was used as negative control.

Antimicrobial susceptibility test

The isolates were screened for their resistance to 5 different antibiotics on Mueller-Hinton (MH) agar using the modified Kirby-Bauer disc diffusion method following the standard guidelines (CLSI, 2016; Talukder et al., 2002). The antibiotics tested were a third-generation cephalosporin, cefotaxime (CTX, 30 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg), tetracycline (TE, 30 µg), azithromycin (AZM, 15 µg) and ciprofloxacin (CIP, 5 µg) (Bio-Rad, USA). *E. coli* ATCC (25922) was used as negative control.

Binary exposure experiment

Based on the results of the MICs of metal salts and antibiotic susceptibility tests, MH agar petri-plates were prepared supplemented with salts of heavy metals, nickel, chromium and lead individually. In this experiment, individual MH agar plates using 0.6 mM $NiSO_4$, 1.0 mM $K_2Cr_2O_7$ and 0.6 mM $PbNO_3$ were prepared for nickel, chromium and lead, respectively. The individual bacterial colony was then spread on MH agar plates supplemented with these individual metal salts using cotton swab and kept at room temperature for 10 minutes. Then antibiotic discs containing 30 µg cefotaxime (CTX), 25 µg sulfamethoxazole-trimethoprim (SXT), 30 µg tetracycline (TE), 15 µg azithromycin (AZM) and 5 µg ciprofloxacin (CIP) (Bio-Rad, USA) were placed on the inoculated plates following standard CLSI guidelines as described (CLSI, 2016; Talukder et al., 2002) and incubated for a period of 24 hours at 37°C. Effects of the presence of metal salts on the spectrum of antibiotic resistance were analyzed based on the values of their zone of inhibition measured in mm scale (Zhou et al., 2015). *E. coli* ATCC (25922) was used as a negative control. Experiments were conducted in triplicate (n=3) to confirm the reproducibility of the experimental results.

Plasmid profiling

The plasmid DNA was extracted according to the simplified alkaline

lysis method developed by Kado and Liu (1981) with minor modifications and subjected to gel electrophoresis using 0.8% agarose in Tris-Borate EDTA buffer (Talukder et al., 2002; Kado and Liu, 1981). In this experiment, reference *E. coli* ATCC (25922) strain does not carry any plasmid and hence was used as negative control. Reference *E. coli* R1 (62 MDa) and V517 (35.6, 4.8, 3.7, 3.4, 3.1, 2, 1.8, 1.4 MDa) strains carry mixture of plasmids and hence were used as positive control (Talukder et al., 2002; Henry, 1991). The molecular weight of the plasmids extracted from the reference strains were converted to molecular size in Kilo base pair (Kb). Molecular weight of the plasmids were determined by comparing them with the 80 to 10,000 bp circular DNA ladder molecular weight markers (Mass Ruler DNA Ladder Mix, Thermo Scientific, USA). Use of these reference negative and positive control strains validated the plasmid extraction protocol as well confirmed the size of the plasmids.

Statistical analysis

Data were analyzed statistically using MS-Excel, R 3.5.3 and RStudio for Windows 10 (32/64 bits). ANOVA with one tail t-test was performed using a linear model to analyze the data and a *p*-value < 0.05 was considered statistically significant. Results of the binary exposure experiment were expressed as mean ± standard deviation (SD) of three replicates.

RESULTS AND DISCUSSION

Total bacterial colony (TBC) count found in effluent samples

After incubation, the plates were observed under a microscope for a five-day period, counting the colonies. Table A in Supplementary information provides the number of colony forming units (CFUs) for each day and for each location (1 and 5) for each sample of a single dilution.

Sample 5 was collected from the drains coming from six different textile industries (Dekko Designs Ltd., Magpie Knit Wear, Hameem Group, AJ Super Garments Ltd., Sharmin Group and Trouser Line Ltd.). In the first day, N, N, 51, 39, 20 CFUs/plate were observed in location 1, 2, 3, 4, 5, respectively, for 10^{-6} dilution. Conversely, for 10^{-10} dilution, on the first day, 7, 12, 6, 9, 2 CFUs/plate were found in location 1, 2, 3, 4, 5 respectively. In day 5, for 10^{-6} dilution, N, 73, 85, 81, N CFUs/plate were observed in location 1, 2, 3, 4, 5, respectively whereas for 10^{-10} dilution, 37, 21, 35, 49, 15 CFUs/plate were counted in location 1, 2, 3, 4, 5, respectively. For other samples, the total CFUs/plate for 10^{-6} to 10^{-10} dilution was presented in Supplementary information (Table A). All other samples had almost identical results (Table 1 and A). The level of CFUs/plate in day 5 was absolutely beyond the allowable standard of CFUs per 2.5 µl volume of effluents for 10^{-6} dilution.

On a standard sized petri-plate, the *E. coli* count is linear and the range is between 30 and 300 CFUs. To make sure that a sample will deliver CFUs in this range, serial dilutions must be performed and then plated. Most standard experimental designs employ ten-fold dilutions (Breed and Dotterrer, 1916), which were also used in this experimental design.

Selection of NLF bacterial colonies

Microscopically and visually, 20 NLF gram-negative colonies were isolated (Figure 1) from five sites (each with four colonies) of one sample. In total, 100 NLF colonies were isolated from five samples (Table 1) where two positive controls, *Shigella flexneri* 2 and *Salmonella serovar* paratyphi B, were used to evaluate colony morphology and to select isolates with type-1 and type-2 NLF colonies, respectively. Type-1 NLF colonies were identified as single, small, round shaped, transparent colonies found on MAC (colorless transparent colonies) or SS (colorless transparent colonies), TCBS (round dark green colonies) and also XLD agar (red colonies) whereas single, well- defined, round, concave shaped, whitish colonies found in MAC (colorless whitish colonies) or SS (colorless whitish with black centered colonies), TCBS (flat dark green colonies) and also XLD (red colored back centered colonies) agar were isolated as type-2 NLF colonies. In total, 50 type-1 and 50 type-2 NLF isolates (counting total 100 isolates) were obtained from five different samples (Table 1) and stored at -4°C for further analysis. In this study, the isolation of *Enterobacteriaceae* ensured contamination of industrial effluents with sewage water. In differential culture plates, the colony morphology can identify the microorganism growing because different microbial species may grow colonies that differ from each other both micro- and macroscopically (*Practical Handbook of Microbiology,* 2015) and this study used colony morphology to identify NLF bacteria.

In a linear model, a normal Q-Q plot was observed for a type-1 isolate and a type-2 NLF isolates (Figure 2). There exists a significant similarity between the two groups of samples (type-1 and type-2) as determined by the ANOVA analysis by having a significant p-value (< 0.05) and a small t-score (0.358773).

Determination of MICs to heavy metals of type-1 and type-2 NLF isolates

MICs (Figure 3) of the 50 type-1 and 50 type-2 NLF bacterial isolates tested for their resistance to 3 different metals where almost all the isolates displayed resistance to these metals tested (Ni, Cr and Pb). In this study, these 3 metals were selected, because these were commonly found metals in waste water (Medfu Tarekegn er al., 2020; Aleem et al., 2003; Murtaza et al., 2002). Figure 3 showed the plate determining MIC of heavy metal where in a single square patch single colony was swabbed.

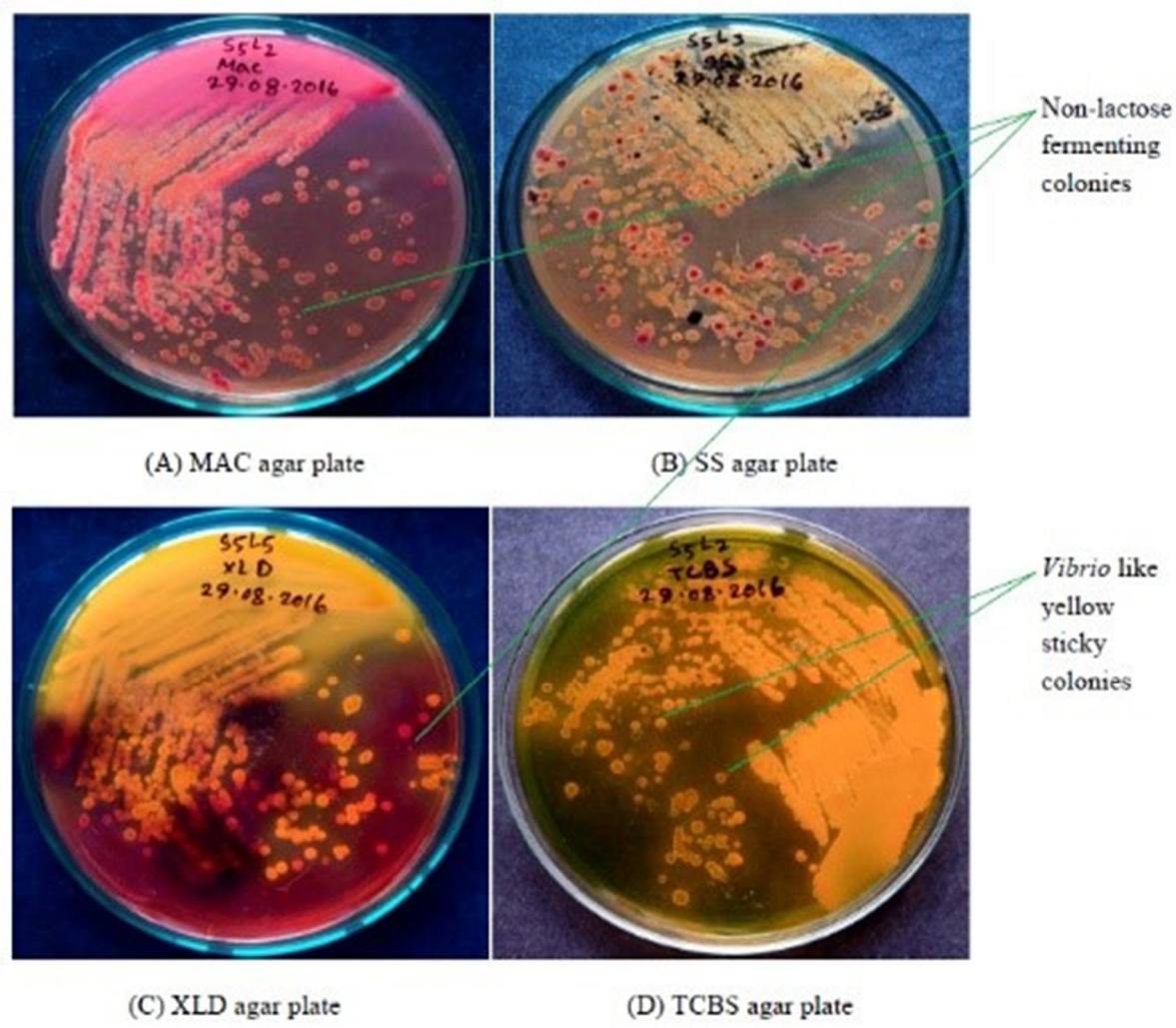

Figure 1. Representative photographs showing morphologies of NLF and *Vibrio* like colonies.

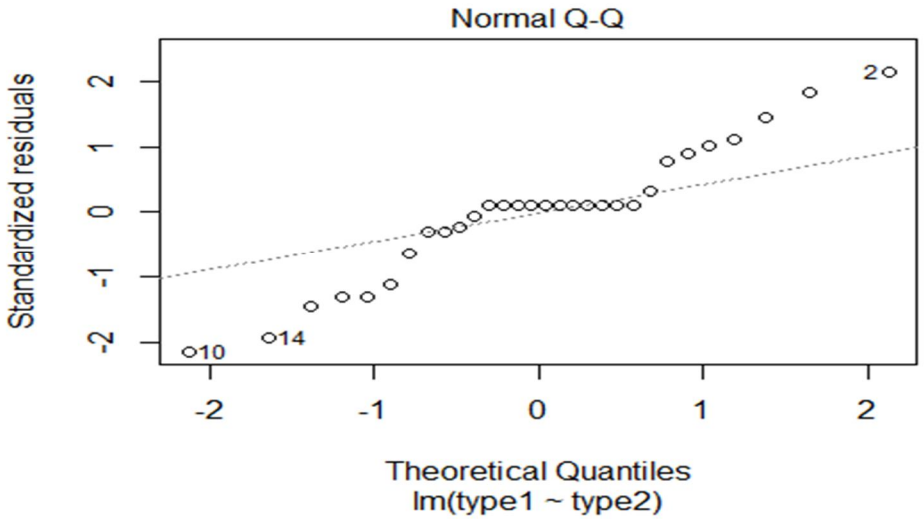

Figure 2. A linear model indicating a normal Q-Q plot for two sets of samples (type-1 and type-2 NLF isolates).

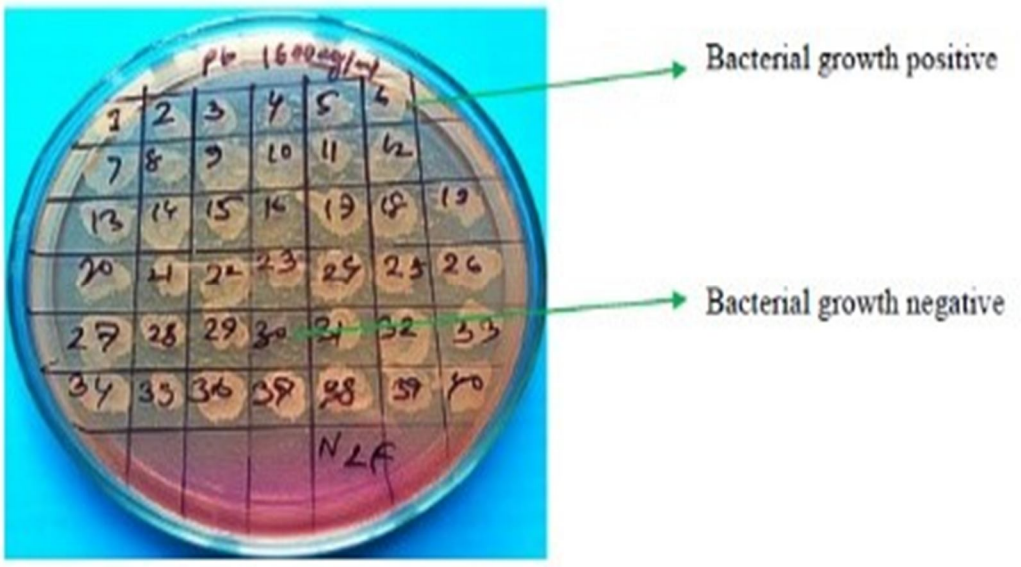

Figure 3. Representative photographs of NLF *Enterobacteriacea* colonies showing MICs of heavy metals determined by plate assay method.

Among 100 NLF isolates, 100% isolates were grown in Ni (0.6 mM), Cr (1.0 mM) and Pb (0.6 mM). Total 59% isolates could tolerate Pb (1.2 mM). Interestingly, 18% isolates, tolerable to Cr (2.0 mM), were obtained from sample 3 (Marma Composites and Fashion Garments Ltd.). Table 2 shows the overall percentage of isolates in different concentrations of heavy metals.

MICs of 50 type-1 NLF isolates

All (n = 50) type-1 NLF isolates had a MIC of 0.6 mM for nickel (Ni) and lead (Pb). For chromium (Cr), 100% (n = 50) isolates had a MIC of 1.0 mM. Only 16% (n = 8) isolates had a MIC of 2.0 mM and 58% (n = 29) isolates had a MIC of 1.2 mM for Cr and Pb, respectively. Significant MICs for 20 type-1 NLF isolates with *E. coli* ATCC (25922) as negative control were given in Table 3.

MICs of 50 type-2 NLF isolates

Among 50 type-2 NLF isolates, for Ni and Pb, 100% (n = 50) isolates had a MIC of 0.6 mM. MICs for Cr were 1.0 mM for 100% of the isolates (n = 50). For Cr and Pb, only 20% of the isolates (n = 10) had MICs of 2.0 mM, and 60% (n = 30) had MICs of 1.2 mM, respectively. Table 4 represents the significant MICs for 20 type-2 NLF isolates with *E. coli* ATCC (25922) as a negative control.

Resistance in bacteria isolated from the textile effluents could be owed to the high concentrations of metals present in the effluents. Resistance to metals and antibiotics was higher in bacteria isolates from metal contaminated areas than in bacteria from a reference site

Table 2. MICs (%) of NLF isolates to heavy metals.

Isolates	Conc. of Ni (mM)				
	0.2	**0.3**	**0.6**	**1.25**	**2.5**
Type-1	100%	100%	100%	0	0
Type-2	100%	100%	100%	0	0
	Conc. of Cr (mM)				
	0.1	**0.3**	**0.5**	**1.0**	**2.0**
Type-1	100%	100%	100%	100%	16%
Type-2	100%	100%	100%	100%	20%
	Conc. of Pb (mM)				
	0.1	**0.2**	**0.4**	**0.6**	**1.2**
Type-1	100%	100%	100%	100%	58%
Type-2	100%	100%	100%	100%	60%

NB: Identification; Conc.: Concentration; mM: Milimolar; Ni: Nickel; Cr: Chromium; Pb: Lead.

(Wright et al., 2006). Aleem et al. (2003) found that 77·1, 71·4, 65·7, 54·2, 45·7 and 25·7% of the bacterial isolated from Aligarh agricultural soil treated with wastewater were resistant to Ni, Cr, cadmium (Cd), zinc (Zn), copper (Cu), and mercury (Hg), respectively. In agreement with the study cited above, the current study results show high metal resistance among bacteria recovered from textile industrial effluents. Murtaza et al. (2002) illustrated that 80 *E. coli* isolates cultured and collected from five different regions of India, 4 regions were contaminated with effluents from household and industry and 1 was from unpolluted area where 68 isolates displayed resistant to at least any one of these metals- Cu, Cd, Pb,

Table 3. MICs of representative type-1 NLF isolates (n=20) to heavy metals.

Lab ID	Heavy metals														
	Nickel (Ni) conc. in mM					Chromium (Cr) conc in mM					Lead (Pb) conc. in mM				
	0.2	0.3	0.6	1.25	2.5	0.1	0.3	0.5	1.0	2.0	0.1	0.2	0.4	0.6	1.6
04	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
05	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
36	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
38	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
40	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
42	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
43	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
44	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-
48	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
49	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
50	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
61	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
63	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
65	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
66	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
67	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
81	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
82	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
88	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
89	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
ATCC 25922	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

NB: ID: Identification; Conc.: Concentration; mM: Milimolar, ATCC (25922): *E.coli* negative control strain; “+”: Growth positive; “-”: Growth negative.

Table 4. MICs of representative type-2 NLF isolates (n=20) to heavy metals.

Lab ID	Heavy metals														
	Nickel (Ni) conc. in mM					Chromium (Cr) conc in mM					Lead (Pb) conc. in mM				
	0.2	0.3	0.6	1.25	2.5	0.1	0.3	0.5	1.0	2.0	0.1	0.2	0.4	0.6	1.6
22	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
24	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
26	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
27	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
29	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
32	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
34	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
51	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
54	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
56	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
57	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
58	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
71	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
74	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
75	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
78	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
80	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
95	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
ATCC 25922	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

NB: ID: Identification; Conc.: Concentration; mM: Milimolar, ATCC 25922: *E. coli* negative control strain; “+”: Growth positive; “-”: Growth negative.

Co, Zn and Fe. Resistance rate of bacteria to heavy metals indicates the severity of environmental contamination by these toxic metals and may be directly linked to their routine exposure to these metals. Alternatively, there is a chance of developing heavy metal resistance in uncontaminated environments or organisms that may be spontaneously accustomed to high toxic levels of metals (Murtaza et al., 2002).

Determination of antimicrobial susceptibility test of NLF isolates (n=100)

To promote animal growth as feed additives and treat human or animal *Enterobacteriaceae* infections, five antibiotics representing different antimicrobial classes were chosen for the experiment (Butler et al., 2020). Overall, among 100 NLF isolates, 32% (n = 32) isolates were multidrug resistant and 35% (n = 35) were intermediary resistant or prone to resistant. It is an interesting fact to mention that 10% (n = 10) multidrug resistant isolates were obtained from sample 4 and 20% multidrug resistant isolates were isolated from sample 2. In Table 5, significant antibiotic susceptibility pattern For 20 type-1 NLF isolates was given and in Table 6, antibiotic susceptibility patterns for 20 type-2 NLF isolates were given including *E. coli* ATCC (25922) as a negative control.

Antimicrobial susceptibility test of type-1 NLF isolates (n=50)

A list of 50 NLF type-1 isolates showed that azithromycin and tetracycline were the most prevalent resistance patterns 38% and 36%, respectively. With ciprofloxacin and sulfamethoxazole-trimethoprim, the percentages of resistance patterns were closer together, estimating 16% and 20%, respectively. Cefotaxime (CTX), with 2% (n = 1) resistance, stood out the least (Figure 4).

Interestingly, 18% (n=9) isolates showed intermediate resistance to CTX and 16% (n = 8) for AZM. There was an intermediate resistance pattern of 10%, 14% and 4%, respectively, for CIP, TE and SXT (Figure 4).

Table 5. Antibiotic susceptibility pattern for representative type-1 NLF isolates (n=20).

Lab ID	Isolate ID	Antibiotics				
		CIP (5µg)	CTX (30µg)	AZM (15µg)	SXT (25µg)	TE (30µg)
04	S1L1 NLF4	S	I	R	S	S
05	S1L2 NLF1	S	I	R	S	S
36	S2L4NLF4	S	S	R	S	R
38	S2L5NLF2	I	I	I	S	R
40	S2L5 NLF4	I	S	I	S	R
42	S3L1NLF2	I	S	S	S	R
43	S3L1NLF3	S	S	S	S	S
44	S3L1NLF4	I	I	S	I	R
48	S3L2NLF4	S	S	S	S	S
49	S3L3NLF1	S	I	S	I	R
50	S3L3 NLF2	S	R	S	S	R
61	S4L1 NLF1	R	S	R	R	R
63	S4L1 NLF3	S	I	I	S	S
65	S4L2NLF1	R	S	R	R	R
66	S4L2NLF2	I	I	S	R	R
67	S4L2NLF3	R	S	R	R	R
81	S5L1NLF1	R	S	R	R	R
82	S5L1NLF2	R	S	R	R	R
88	S5L2NLF4	R	S	S	R	R
89	S5L3NLF1	R	S	S	R	R
ATCC	25922	S	S	S	S	S

NB: ID: Identification; NLF1: Non-lactose fermenter 1; NLF2: Non-lactose fermenter 2; NLF3: Non-lactose fermenter 3; NLF4: Non-lactose fermenter 4; CIP: Ciprofloxacin; CTX: Cefotaxime; AZM: Azithromycin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline; S: Sensitive; I: Intermediary; R: Resistance.

Table 6. Antibiotic susceptibility pattern for representative type-2 NLF isolates (n=20).

Lab ID	Isolate ID	Antibiotics				
		CIP (5µg)	CTX (30 µg)	AZM (15 µg)	SXT (25 µg)	TE (30 µg)
22	S2L1 NLF2	I	S	R	S	R
24	S2L4NLF4	R	S	R	R	R
26	S2L2 NLF2	S	I	I	S	R
27	S2L2NLF3	S	I	R	S	R
29	S2L3 NLF1	I	S	R	S	R
32	S2L3NLF4	I	S	R	S	R
34	S2L4NLF2	S	S	R	S	R
51	S3L3NLF3	S	I	S	I	I
54	S3L4NLF2	S	R	S	S	S
56	S3L4NLF4	S	I	S	I	S
57	S3L5 NLF1	S	I	S	S	I
58	S3L5 NLF2	I	I	S	I	R
71	S4L3 NLF3	R	S	R	R	R
74	S4L4 NLF2	S	R	S	S	I
75	S4L4 NLF3	S	S	S	S	S
78	S4L5 NLF2	R	S	R	R	R
80	S4L5 NLF4	S	R	R	S	S
95	S5L4 NLF3	S	S	S	S	S
ATCC	25922	S	S	S	S	S

NB: ID: Identification; NLF1: Non-lactose fermenter 1; NLF2: Non-lactose fermenter 2; NLF3: Non-lactose fermenter 3; NLF4: Non-lactose fermenter 4; CIP: Ciprofloxacin; CTX: Cefotaxime; AZM: Azithromycin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline; S: Sensitive; I: Intermediary; R: Resistance.

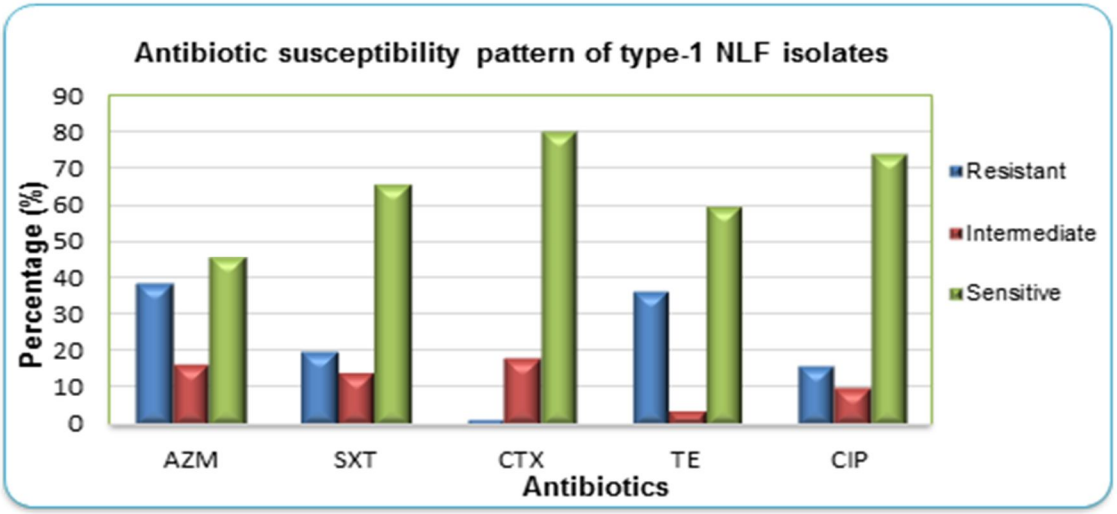

Figure 4. Distribution (%) of antibiotic susceptibility pattern of representative type-1 NLF isolates (n=50) (CIP: Ciprofloxacin; CTX: Cefotaxime; AZM: Azithromycin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline).

Antimicrobial susceptibility test of type-2 NLF isolates

Among 50 type-2 NLF isolates, most prevalent (48%, n = 24) resistance pattern was found for azithromycin (AZM) followed by 44% (n = 22) for tetracycline (TE). For ciprofloxacin (CIP) and sulfamethoxazole-trimethoprim (SXT), the percentages for resistance pattern were equal to each other, estimating 10% (n = 5) each. The lowest resistance pattern was found for cefotaxime (CTX),

counting 8% (n = 4) (Figure 5).

It is interesting to note that 20% (n = 10) isolates were showing intermediary resistance pattern to CTX and 2% (n = 1) for AZM. For CIP, TE and SXT, the intermediary resistance pattern was found 18% (n = 9), 8% (n = 4) and 10% (n = 5), respectively (Figure 5). So, it is possible that all these intermediary resistant isolates would turn into complete resistant within a short period of time.

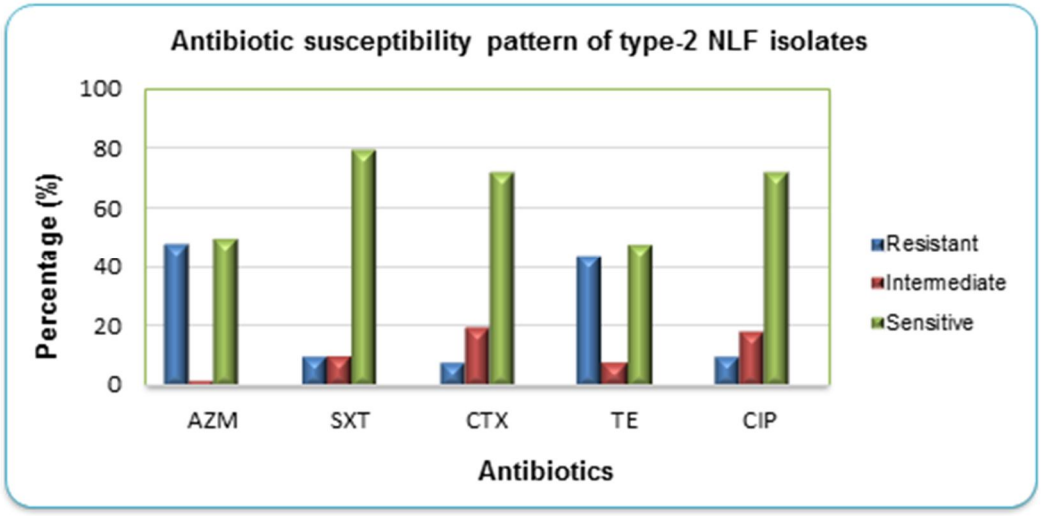

Figure 5. Distribution (%) of antibiotic susceptibility pattern of representative type-2 NLF isolates (n=50) (CIP: Ciprofloxacin; CTX: Cefotaxime; AZM: Azithromycin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline).

It has been demonstrated by the isolation of antibiotic resistant *Enterobacteriaceae* that industrial effluents were contaminated with nearby wastewater and that antibiotics were being misused by local residents and manufacturing workers. Heavy metal and antibiotic resistance was observed in bacteria isolated from the soil of agricultural land exposed continuously to contaminated waste water (Wright et al., 2006). The results of this study were in agreement with those from earlier studies.

Metal contamination directly selects metal-tolerant bacteria while co-selecting antibiotic-tolerant bacteria (Wright et al., 2006). Resistance or tolerance to the hazardous heavy metals is clearly correlated with multi-resistant bacteria in environmental samples. The heavy metals and drugs including antibiotics that have been exposed to the environment as a result of human-induced pollution have been found to exert a selective pressure for resistance buildup in bacteria (Lazăr et al., 2002). This study confirmed the findings of the previous studies. Industrial environments are regularly contaminated with a variety of organic and inorganic pollutants (Aleem et al., 2003; Aleem and Malik, 2003; Ansari and Malik, 2007). Bacteria from contaminated soils (Máthé et al., 2012), surface water (Koc et al., 2013) and even the shallow sediments of Antarctica (Lo Giudice et al., 2013), have also been reported to display cross-resistance to toxic heavy metals and antibiotics. Detailed mechanisms of cross-resistance between heavy metals and antibiotics are still unclear.

Binary exposure experiment of representative NLF isolates (n=13)

A binary exposure experiment measured the effects of heavy metals on antibiotic zones of inhibition. In this experiment, significant 5 type-1 and 8 type-2 NLF isolates were selected based on MIC and antimicrobial susceptibility test results. A selection of isolates was those with at least one antibiotic resistance, either full or intermediate. Each of the three types of MH agar plates was prepared adding Ni (0.6 mM), Cr (1.0 mM), and Pb (0.6 mM). The metal concentrations were determined based on the highest isolates that could tolerate that specific concentration. Among 4 type-1 NLF isolates, all were able to tolerate these specific concentrations, while three could tolerate Cr and Pb. Five out of seven type-2 NLF isolates were able to tolerate Pb, and four were able to tolerate both Cr and Ni. The two other isolates were taken from type-1 and type-2 NLF isolates, both of which were susceptible to all antibiotics, but could still withstand these specific metal concentrations. Table 7 included the mean value of zone diameter with standard deviations.

Overall, Table 7 indicated that the antibiotic inhibition zone diameter increased in most cases in the presence of heavy metals, decreased in some cases, and did not change in a few cases. Because of this finding, it is plausible that these metal resistant isolates, with negative or no effects on antibiotic resistance could be applied for bioremediation. Medfu Tarekegn et al. (2020) found that

Table 7. Zone of inhibition of the representative type-1 and type-2 NLF isolates to the heavy metals, Ni (0.6 mM), Cr (1.0 mM), Pb (0.6 mM), respectively in the presence of antibiotics.

Lab ID	AZM (diameter in mm)		SXT (diameter in mm)		CTX (diameter in mm)		TE (diameter in mm)		CIP (diameter in mm)	
	Ab	Ab + HM	Ab	Ab + HM	Ab	Ab + HM	Ab	Ab + HM	Ab	Ab + HM
Type-1 NLF isolates										
44	18±0.32	(Ni) 22±0.46 (Cr) - (Pb) -	14±0.24	(Ni) 30±0.64 (Cr) - (Pb) -	16±0.33	(Ni) 20±0.42 (Cr) - (Pb) -	12±0.18	(Ni) 20±0.44 (Cr) - (Pb) -	18±0.28	(Ni) 20±0.35 (Cr) - (Pb) -
61	7±0.06	(Ni) 7±0.09 (Cr) 7±0.06 (Pb) 7±0.07	7±0.06	(Ni) 7±0.03 (Cr) 7±0.07 (Pb) 7±0.06	28±0.82	(Ni) 30±0.66 (Cr) 30±0.75 (Pb) 22±0.58	7±0.06	(Ni) 7±0.03 (Cr) 7±0.06 (Pb) 7±0.05	7±0.06	(Ni) 7±0.05 (Cr) 7±0.04 (Pb) 7±0.08
65	7±0.05	(Ni) 7±0.04 (Cr) 7±0.12 (Pb) 7±0.08	7±0.09	(Ni) 7±0.08 (Cr) 7±0.11 (Pb) 7±0.04	26±0.66	(Ni) 28±0.64 (Cr) 30±0.76 (Pb) 30±0.84	7±0.04	(Ni) 7±0.04 (Cr) 7±0.06 (Pb) 7±0.08	7±0.08	(Ni) 7±0.07 (Cr) 7±0.03 (Pb) 7±0.05
81	7±0.08	(Ni) 7±0.05 (Cr) 7±0.07 (Pb) 7±0.13	7±0.04	(Ni) 7±0.12 (Cr) 7±0.05 (Pb) 7±0.06	30±0.75	(Ni) 30±0.85 (Cr) 30±0.84 (Pb) 32±0.68	14±0.28	(Ni) 18±0.26 (Cr) 7±0.05 (Pb) 7±0.07	7±0.04	(Ni) 7±0.06 (Cr) 7±0.04 (Pb) 7±0.08
48*	20±0.53	(Ni) 24±0.55 (Cr) 19±0.36 (Pb) 27±0.64	20±0.42	(Ni) 21±0.35 (Cr) 20±0.22 (Pb) 24±0.43	24±0.46	(Ni) 28±0.65 (Cr) 21±0.54 (Pb) 29±0.63	20±0.42	(Ni) 24±0.26 (Cr) 18±0.22 (Pb) 26±0.48	26±0.35	(Ni) 30±0.58 (Cr) 22±0.36 (Pb) 28±0.73
Type-2 NLF isolates										
24	7±0.04	(Ni) 7±0.05 (Cr) 30±0.66 (Pb) -	7±0.05	(Ni) 22±0.52 (Cr) 7±0.03 (Pb) -	28±0.57	(Ni) 30±0.88 (Cr) 10±0.15 (Pb) -	7±0.05	(Ni) 7±0.05 (Cr) 30±0.85 (Pb) -	7±0.09	(Ni) 7±0.07 (Cr) 30±0.72 (Pb) -
51	24±0.51	(Ni) 26±0.56 (Cr) - (Pb) -	14±0.33	(Ni) 22±0.32 (Cr) - (Pb) -	18±0.36	(Ni) 24±0.36 (Cr) - (Pb) -	18±0.21	(Ni) 22±0.36 (Cr) - (Pb) -	22±0.41	(Ni) 28±0.67 (Cr) - (Pb) -
54	20±0.55	(Ni) 7±0.08 (Cr) - (Pb) -	18±0.28	(Ni) 26±0.56 (Cr) - (Pb) -	14±0.22	(Ni) 30±0.64 (Cr) - (Pb) -	20±0.38	(Ni) 18±0.18 (Cr) - (Pb) -	24±0.38	(Ni) 20±0.46 (Cr) - (Pb) -
71	7±0.06	(Ni) 7±0.05 (Cr) 7±0.07 (Pb) 7±0.11	7±0.04	(Ni) 7±0.12 (Cr) 7±0.04 (Pb) 7±0.11	26±0.59	(Ni) 28±0.55 (Cr) 30±0.62 (Pb) 32±0.74	12±0.18	(Ni) 16±0.21 (Cr) 7±0.05 (Pb) 7±0.07	7±0.05	(Ni) 7±0.06 (Cr) 7±0.03 (Pb) 7±0.05

Table 7. Continues.

74	20±0.48	(Ni) 24±0.45 (Cr) 26±0.54 (Pb) 24±0.52	16±0.31	(Ni) 20±0.36 (Cr) 20±0.24 (Pb) 22±0.28	10±0.15	(Ni) 32±0.83 (Cr) 28±0.74 (Pb) 30±0.62	18±0.24	(Ni) 24±0.27 (Cr) 30±0.56 (Pb) 26±0.63	28±0.68	(Ni) 32±0.74 (Cr) 22±0.64 (Pb) 22±0.48
78	7±0.07	(Ni) - (Cr) 7±0.06 (Pb) 7±0.09	7±0.02	(Ni) - (Cr) 7±0.06 (Pb) 7±0.08	26±0.64	(Ni) - (Cr) 30±0.58 (Pb) 30±0.82	10±0.12	(Ni) - (Cr) 7±0.04 (Pb) 12±0.08	7±0.03	(Ni) - (Cr) 7±0.03 (Pb) 7±0.07
80	7±0.05	(Ni) - (Cr) 7±0.07 (Pb) 7±0.05	16±0.34	(Ni) - (Cr) 7±0.02 (Pb) 7±0.10	14±0.28	(Ni) - (Cr) 28±0.58 (Pb) 30±0.82	20±0.36	(Ni) - (Cr) 7±0.06 (Pb) 14±0.12	24±0.28	(Ni) - (Cr) 7±0.09 (Pb) 7±0.04
75*	22±0.44	(Ni) 26±0.53 (Cr) 20±0.41 (Pb) 24±0.54	22±0.49	(Ni) 24±0.24 (Cr) 20±0.37 (Pb) 22±0.41	30±0.65	(Ni) 32±0.68 (Cr) 28±0.74 (Pb) 32±0.64	30±0.74	(Ni) 26±0.38 (Cr) 30±0.65 (Pb) 30±0.73	24±0.42	(Ni) 28±0.62 (Cr) 20±0.74 (Pb) 26±0.56
***E. coli* ATCC**	28±0.61	(Ni) 25±0.48 (Cr) 22±0.51 (Pb) 23±0.44	28±0.53	(Ni) 26±0.58 (Cr) 23±0.60 (Pb) 24±0.55	30±0.71	(Ni) 28±0.58 (Cr) 26±0.62 (Pb) 25±0.48	30±0.62	(Ni) 27±0.64 (Cr) 25±0.54 (Pb) 26±0.77	28±0.36	(Ni) 26±0.74 (Cr) 25±0.75 (Pb) 23±0.54

NB: ID: Identification; Mean ± Standard deviation; CIP: Ciprofloxacin; CTX: Cefotaxime; AZM: Azithromycin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline; Ab: Antibiotic; HM: Heavy metal; mm: Millimeter; Ni: Nickel; Cr: Chromium; Pb: Lead; 48*: Type-1 antibiotic sensitive isolate; 75*: Type-2 antibiotic sensitive isolate;ATCC (25922): Negative control strain.

these isolate can accumulate metals into their bodies, and are not harmful for people and animals, if these isolate discharge into the environment.

This study concluded that Cr and Pb had the most significant influence on CTX, SXT and TE, as well as Ni on TE, AZM, and CIP since at least one isolate demonstrated a decreased zone of inhibition causing potentially toxic effects if discharged into the environment. In the presence of Ni, Cr, and Pb the zone diameter decreased for all antibiotics in *E. coli* ATCC (25922). The complex study findings were dependent on the types and concentrations of antibiotics and the heavy metals used. There might be two aspects to these findings, the chemical reactions between heavy metals and antibiotics and their individual bio-effects. The chemical reactions between them depend on the concentrations in the system of heavy metals, antibiotics and other pollutants (Zhang et al., 2012; Alonso et al., 2001).

It is possible that toxic products from the chemical reaction would weaken the bacterial tolerance to antibiotics (Tamilselvi and Mugesh, 2008). Nevertheless, the effects reverse or remain unchanged if the toxicity of the products is less than that of the parent substance. In *Pseudomonas aeruginosa*, it is possible that resistance to imipenem is greatly enhanced by Zn and Cu, because of possible coagulation (Caille et al., 2007). These results support the observations of McArthur and Tuckfield (2000), where a positive correlation between antibiotic resistance and Hg concentration was found in the stream after the antibiotic-sensitive bacteria were discharged. Such evidence implies that this metal could contribute to the proliferation of antibiotic-resistant bacteria. An in-depth understanding of the dynamics and ecology of these bacterial populations, as well as the natural selective pressure factors, will provide a comprehensive view of how antibiotic resistance evolves in populations (McArthur and Tuckfield, 2000).

Cross-resistance to antibiotics can be induced by heavy metal resistance. Heavy metals in trace amounts are essential for protein and enzyme activity to maintain bacteria's growth in general (Liu et al., 2012), but in *Psedomonas fluorescens*

stressing amounts adversely affected the synthesis of proteins (Sharma et al., 2006). Some types of stress can affect cross-resistance mechanisms, such as efflux pumps and integron-containing mobile elements and enabling a synergetic resistance to both Cr and some antibiotics (Petrova et al., 2011). *Vibrio* species carry cryptic plasmids that have an ecological significance in their resistance to Hg and antibiotics (Wang et al., 2006; Zhang et al., 2006). Moreover, the expression of metallothioneins was induced by elevated levels of certain metals, and as a result, antibiotic resistance was inhibited rather than enhanced (Strouhal et al., 2003).

Despite the recently recognized role of heavy metals in bacterial antibiotic resistance, the exact mechanisms remain unclear due to a lack of evidence about synchronized resistance to heavy metals and antibiotics in microbes. A chronometric study on the cross-resistance of a given bacterium may reveal environmental risk factors for heavy metals and antibiotics.

Plasmid profiling of type-1 and type-2 NLF isolates (n=13)

A plasmid profiling (Figure 6) analysis revealed various plasmid patterns from P1 to P6 (Table 8). Three plasmid patterns were found among 5 type-1 isolates: P1, P2 and P3. In three isolates, no plasmid was found including the antibiotic-susceptible isolate. One isolate contained a plasmid with a large size (14Kb) and the other isolate had small plasmids ranging from 4 to 1.8 Kb. Two isolates resistant to four antibiotics (AZM, SXT, CIP, and TE) had no plasmid as did the antibiotic sensitive isolate.

Among 8 type-2 isolates, four plasmid patterns (P2, P4, P5 and P6) were identified. Those isolate had the same plasmid pattern, with six plasmids, one of which was 13 kb. One more isolate contained a plasmid in the range of 13 Kb while two isolates were 12 Kb. Three isolates were plasmid less, including one isolate that was antibiotic-susceptible. The *E. coli* ATCC (25922) strain was plasmid-less as well. It is very interesting that two isolates, one resistant to CTX and another resistant to both CTX and AZM, did not have any plasmid as sensitive isolate Table 8 contains the plasmid size and patterns of the eight most significant types-1 isolates and four types-2 isolates. Table 9 provides a summary of the characteristics of the 14 most significant isolates, including two susceptible isolates as well as one negative control.

The high incidence of metal resistant populations and the presence of plasmid-bearing strains is frequently found in polluted sites compared with unpolluted sites, as Malik and Jaiswal suggested in 2000. Most of the bacteria isolated for the present study had significant resistance to the tested antibiotics and heavy metals. There is now no doubt that resistance genes reside in plasmids (Collard et al., 1994; Dhakephalkar and Chopade, 1994; Guo et al., 2006). Often, the Extended Spectrum β-Lactamase-producers have a multidrug-resistant plasmid that confers resistance to both β-lactam and non- β-lactam antibiotics (Xiong et al., 2002). Nevertheless, our present study shows that CTX-resistant isolates contained no plasmid, although they were a β -lactamase producers.

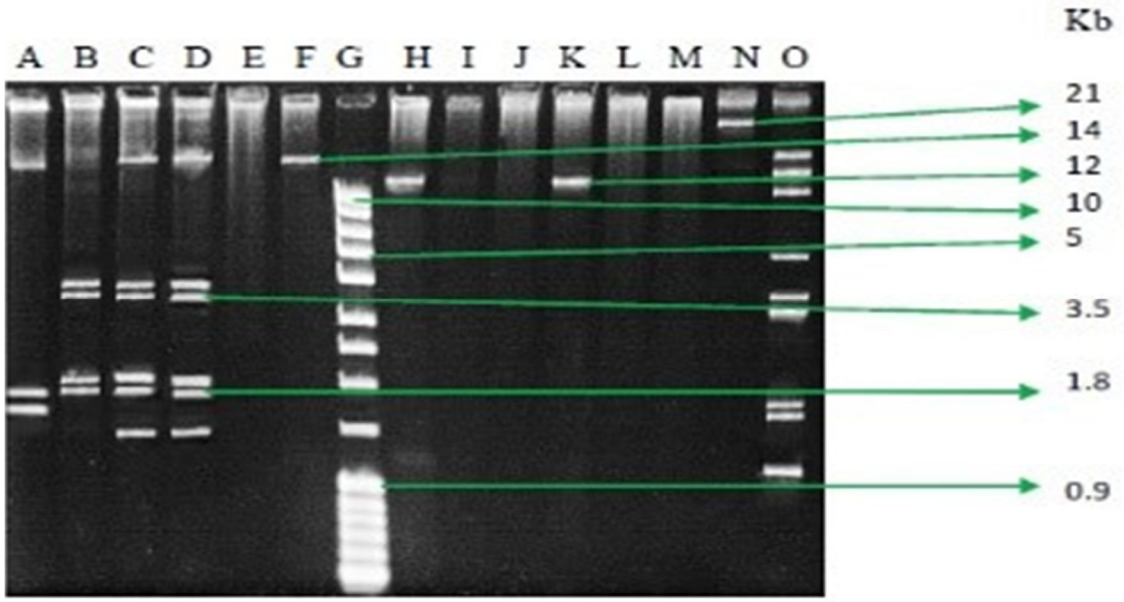

Figure 6. Plasmid profiles of representative type-1 (n=4) and type-2 (n=8) NLF isolates (Lane A: 24 (type-2); Lane B: 44 (type-1), Lane C: 51 (type-2), Lane D: 54 (type-2), Lane E: 61 (type-1), Lane F: 65 (type-1); Lane G: Circular DNA Ladder; Lane H: 71 (type-2), Lane I: 74 (type-2), Lane J: 75 (antibiotic sensitive type-2); Lane K: 78 (type-2), Lane L: 80 (type-2), Lane M: 81 (type-1), Lane N: R1, Lane O: V-517).

Table 8. Plasmid patterns of type-1 (n=5) and type-2 (n=8) NLF isolates showing resistance to both heavy metals and antibiotics.

Lab ID	Isolate ID	Lanes in Figure 6	Plasmid size in Kilo base pair (Kb)	Plasmid pattern (P)
Type-1 NLF isolates				
44	S3L1 NLF4	Lane B	4,3.5,2,1.8	P1
61	S4L1 NLF1	Lane E	Plasmid less	P2
65	S4L2 NLF1	Lane F	14	P3
81	S5L1 NLF1	Lane M	Plasmid less	P2
48*	S3L2 NLF4		Plasmid less	P2
Type-2 NLF isolates				
24	S2L4 NLF4	Lane A	13,1.7,1.6	P4
51	S3L3NLF3	Lane C	13,4,3.5,2,1.8,1.5	P5
54	S3L4NLF2	Lane D	13,4,3.5,2,1.8,1.5	P5
71	S4L3 NLF3	Lane H	12	P6
74	S4L4 NLF2	Lane I	Plasmid less	P2
78	S4L5 NLF2	Lane K	12	P6
80	S4L5 NLF4	Lane L	Plasmid less	P2
75*	S4L4 NLF3	Lane J	Plasmid less	P2
***E. coli* marker strains**				
	ATCC 25922		Plasmid Less	P2
	R 1	Lane N	21	
	V 517	Lane O	14,12,10,5,3.5,3,1.7,1.6,1	

NB: ID: Identification; 48*: Type-1 antibiotic sensitive isolate; 75*: Type-2 antibiotic sensitive isolate; R1: Reference strain; V517: Reference strain; P: Pattern.

Table 9. Representative type-1 and type-2 NLF isolates (n=14) showing susceptible pattern to heavy metals and antibiotics in relation to their plasmid profiles.

Lab ID	Isolate ID	Antibiotic susceptibility test			Maximum tolerable conc. to heavy metals (mM)			In the presence of maximum tolerable conc. of heavy metal, the changes of zone of inhibition of antibiotics measured in mm						Plasmid profiling (size in Kb)
								Ni (0.6 mM)			Cr (1.0 mM)		Pb (0.6 mM)	
		R	I	S	Ni	Cr	Pb	E	D	E	D	E	D	
Type-1 NLF isolates														
44	S3L1NLF4	TE	SXT,CTX,CIP	AZM	0.6	2.0	1.2	AZM,SXT,CTX,TE,CIP	-	-	-	-	-	4,3.5,2,1.8
61	S4L1NLF 1	AZM,SXT,TE,CIP	-	CTX	0.6	1.0	0.6	CTX	-	CTX	-	CTX,TE	-	Plasmid less
65	S4L2NLF1	AZM,SXT,TE,CIP	-	CTX	0.6	1.0	0.6	CTX	-	CTX	-	CTX		14
81	S5L1NLF1	AZM,SXT,TE,CIP	-	CTX	0.6	1.0	0.6	TE	-	-	TE	CTX	TE	Plasmid less
48*	S3L2NLF4	-	-	All	0.6	1.0	0.6	-	-	-	-	-	-	Plasmid less

Table 9. Continues.

Type-2 NLF isolates														
24	S2L1NLF4	AZM,SXT,TE,CIP	-	CTX	0.6	1.0	1.2	SXT,CTX	-	AZM,TE,CIP	CTX	-	-	13,1.7,1.6
51	S3L3NLF3	CTX	SXT,TE	AZM,CIP	0.6	2.0	1.2	AZM,SXT,CTX, TE,CIP	-	-	-	-	-	13,4,3.5,2,1.8,1.5
54	S3L4NLF2	CTX	-	AZM,SXT, TE,CIP	0.6	2.0	1.2	SXT,CTX	AZM,TE,CIP	-	-	-	-	13,4,3.5,2,1.8,1.5
71	S4L3NLF3	AZM,SXT,TE,CIP		CTX	0.6	1.0	0.6	CTX,TE	-	CTX	TE	CTX	TE	12
74	S4L4NLF 2	CTX	TE	AZM,SXT, CIP	0.6	1.0	0.6	AZM,SXT,CTX, TE,CIP	-	AZM,SXT, CTX, TE	CIP	AZM,SXT, CTX, TE	CIP	Plasmid less
78	S4L5NLF2	AZM,SXT,TE,CIP	-	CTX	0.3	1.0	0.6	-	-	CTX	TE	CTX,TE		12
80	S4L5NLF4	AZM,CTX	-	SXT,TE, CIP	0.3	1.0	0.6	-	-	CTX	SXT,TE, CIP	CTX	SXT,TE, CIP	Plasmid less
75*	S4L4NLF3	-	-	All	0.6	1.0	0.6	AZM,SXT,CTX, CIP	TE	-	AZM,SXT, CTX, CIP	AMZ,CTX, CIP	-	Plasmid less
Negative control *E. coli*														
E. coli	ATCC (25922)	-	-	All	-	-	-	-	-	-	-	-	-	Plasmid less

NB: ID: Identification; R: Resistance, I: Intermediate, S: Sensitive, CIP: Ciprofloxacin; CTX: Cefotaxime; AZM: Azithromycin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline; Conc.: Concentration; Ni: Nickel; Cr: Chromium; Pb: Lead; E: Enhanced; D: Decreased, Kb: Kilobasepair, 48*: Type-1 antibiotic sensitive isolate, 75*: Type-2 antibiotic sensitive isolates, ATCC (25922): *E. coli* negative control strain.

Previous studies have shown that the multidrug resistance plasmids generally range a sized of ≥12 Kb (Jacoby and Sutton, 1991; Parvin et al., 2014). In a similar way, our study had the same results, because several significant bacterial isolates found in textile effluents had resistance to various antibiotics and heavy metals and also showed evidence of plasmid DNA of sizes 13 Kb, 14 Kb and 12 Kb. Our results support previous findings in the presence of heavy metal resistant bacteria with plasmids in heavy metal contaminated environment (Vajiheh et al., 2003; Zou Algharneyn et al., 2007).

In our study, we also found that one TE resistant isolate had no plasmid. Thus, it was found that plasmid-free bacteria could exhibit either antibiotic or heavy metal resistance, clearly demonstrating there was no consistent association between plasmid profiles and antibiotic/heavy metal resistance. Some antibiotic/ heavy metal resistance properties are correlated with plasmids, chromosomal DNA, transposons and phages (Jain et al., 2009; Vajiheh et al., 2003), which are inadequate to determine the relationship between antibiotic/ heavy metal resistance and polluted environment and prompt an erroneous epidemiologic conclusion.

CONCLUSION

Monitoring the quality of the industrial effluents and sewage water is very important since the effluents eventually discharge into the rivers. Our present study indicates that industrial effluents are organically enriched media, supporting fast growth and spreading of microbes resistant to multiple antibiotics and metallic ions. A notable health hazard of antibiotic abuse or heavy metal pollution is the proliferation of these antibiotic-resistant bacteria exerting therapeutic failure, although the heavy metals in most cases have negative or no effect on antibiotic resistance. Thus, the resistance to metal ions presently represents less clinical concern; however, the plasmid genetics, physiology, and antibiotic resistance mechanisms of microbial communities in polluted environments could be better understood by the knowledge of heavy metal resistance mechanisms. It is also likely to mention that if heavy metal resistant isolates are non-hazardous, they also could be used for bioremediation to treat industrial effluents, after testing their biosorption or bioaccumulation efficiency of heavy metals.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Supplementary information

Table A. Day wise breakup of total bacterial colony (TBC) counts at varying dilution.

Sample ID	Plate no.	Dilution factor	Total bacterial colony (TBC) count by plate observation				
			Day1 CFU/plate	Day2 CFU/plate	Day3 CFU/plate	Day4 CFU/plate	Day5 CFU/plate
S1L1	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	N	N	N	N	N
	3	10^{-8}	N	N	N	N	N
	4	10^{-9}	51	59	63	74	N
	5	10^{-10}	27	33	48	52	N
S1L5	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	47	51	60	66	71
	3	10^{-8}	39	45	55	57	63
	4	10^{-9}	24	31	43	49	56
	5	10^{-10}	11	16	21	26	35
S2L1	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	N	N	N	N	N
	3	10^{-8}	12	33	37	41	N
	4	10^{-9}	11	29	31	35	45
	5	10^{-10}	7	23	28	32	37
S2L5	1	10^{-6}	20	42	N	N	N
	2	10^{-7}	11	29	N	N	N
	3	10^{-8}	9	14	17	19	24
	4	10^{-9}	8	13	15	20	22
	5	10^{-10}	2	4	5	8	15
S3L1	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	N	N	N	N	N
	3	10^{-8}	N	N	N	N	N
	4	10^{-9}	11	14	16	19	N
	5	10^{-10}	7	9	16	19	23
S3L5	1	10^{-6}	53	57	61	77	83
	2	10^{-7}	45	48	50	56	63
	3	10^{-8}	31	37	44	49	51
	4	10^{-9}	23	26	31	37	41
	5	10^{-10}	13	19	23	28	32
S4L1	1	10^{-6}	42	45	48	N	N
	2	10^{-7}	36	39	43	N	N
	3	10^{-8}	24	27	33	46	N
	4	10^{-9}	12	18	21	38	N
	5	10^{-10}	7	8	15	23	N
S4L5	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	N	N	N	N	N
	3	10^{-8}	2	5	8	N	N
	4	10^{-9}	2	3	5	N	N
	5	10^{-10}	1	2	4	9	N
S5L1	1	10^{-6}	69	N	N	N	N
	2	10^{-7}	40	N	N	N	N
	3	10^{-8}	23	23	30	N	N
	4	10^{-9}	10	13	13	16	18
	5	10^{-10}	4	8	10	14	17
S5L5	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	15	21	27	38	53

Table A. Continues.

3	10^{-8}	9	16	16	18	41
4	10^{-9}	8	13	14	21	35
5	10^{-10}	6	18	20	23	27

NB: S1L1: Sample 1 location; S1L5: Sample 1 location 5; S2L1: Sample 2 location 1; S2L5: Sample 2 location 5; S3L1: Sample 3 location 1; S3L5: Sample 3 location 5; S4L1: Sample 4 location 1; S4L5: Sample 4 location 5; S5L1: Sample 5 location 1; S5L5: Sample 5 location 5; N: Numerous; ID: Identification; CFU: Colony forming unit.