Saudi Pharmaceutical Journal 28 (2020) 1626–1634

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

Pharmaceutical disposal facilitates the mobilization of resistance determinants among microbiota of polluted environment

Mudsser Azam [a,1], Vijay Kumar [b,1], Kehkashan Siddiqui [a], Arif Tasleem Jan [c], Jamal S.M. Sabir [d,e], Irfan A. Rather [d,e,*], Suriya Rehman [f,*], Qazi Mohd Rizwanul Haq [a,*]

[a] *Department of Biosciences, Jamia Millia Islamia, New Delhi, India*
[b] *Department of Biotechnology, Yeungnam University, Gyeongsan, Republic of Korea*
[c] *School of Biosciences and Biotechnology, Baba Ghulam Shah Badshah University, Rajouri, India*
[d] *Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah -21589, Saudi Arabia*
[e] *Center of Excellence for Bionanoscience Research, King Abdulaziz University, Jeddah -21589, Saudi Arabia*
[f] *Department of Epidemic Disease Research, Institute for Research and Medical Consultation (IRMC), Imam Abdulrahman Bin Faisal University, Dammam, 31441 Dammam, Saudi Arabia*

A R T I C L E   I N F O

Article history:
Received 19 July 2020
Accepted 18 October 2020
Available online 28 October 2020

Keywords:
Multidrug resistance
Resistance prevalence
ESBLs
E. coli
Conjugation frequency
Dissemination

A B S T R A C T

The emergence of resistance on exposure to pharmaceuticals among microorganisms has raised serious concern in the therapeutic approach against infectious diseases. Effluents discharge from hospitals, industries, and urban settlements containing pharmaceuticals and other toxic compounds into the aquatic ecosystem selects bacterial population against them; thereby promotes acquisition and dissemination of resistant traits among the inhabitant microbiota. The present study was aimed to determine the prevalence and multidrug resistance pattern of Extended Spectrum β-lactamase (ESBL) producing and non-producing bacterial isolates from the heavily polluted Delhi stretch of river Yamuna, India. Additionally, the role of abiotic factors in the dissemination of conjugative plasmids harbouring resistance genes was also studied using *E. coli* J53 as recipient and resistant *E. coli* isolates as donor strains. Of the 227 non-duplicate bacterial isolates, 60% (136) were identified as ESBL$^+$ and 40% (91) as ESBL. ESBL$^+$ isolates were found highly resistant to β-lactam and non-β-lactam classes of antibiotics compared with the ESBL$^-$ isolates. 68% of ESBL$^+$ and 24% of ESBL$^-$ isolates showed an MAR index of $\geq$0.5. Surprisingly, multidrug resistance (MDR), extensively drug resistance (XDR), and pandrug resistance (PDR) phenotype were observed for 78.6%, 16.9%, and 0.7% of ESBL$^+$ and 90%, 3%, and none for PDR among ESBL$^-$ isolates. Conjugation under different conditions showed a higher mobilization rate at neutral pH (7–7.5) for ESBL$^+$ isolates. Conjugation frequency was maximum at 40 °C for the isolate *E. coli* MRB6 (4.1×10^{-5}) and *E. coli* MRE32 (4.89×10^{-4}) and at 35 °C for *E. coli* MRA11 (4.89×10^{-5}). The transconjugants obtained were found tolerating different concentrations of mercuric chloride (0.0002–0.2 mg/L). Increased biofilm formation for ESBL$^+$ isolates was observed on supplementing media with $HgCl_2$ (2 μg/mL) either singly or in combination with CTX (10 μg/mL). The present study demonstrates that anthropogenically influenced aquatic environments act as a reservoir of MDR, XDR, and even PDR strains; thereby posing a potent public health risk.

© 2020 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The emergence of multidrug resistance among bacteria has largely compromised the treatment of infections (Davey et al., 2011; Bassetti et al., 2019). Bacteria deploy mechanisms like drug inactivation, target site alteration, efflux pump, and their combinations, to evade the effect of antimicrobial compounds. Among the different modes, resistance acquired through the production of β-lactamases that subsequently emerged with different allelic

* Corresponding authors at: Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia (I.A. Rather); Imam Abdulrahman Bin Faisal University, Dammam, 31441 Dammam, Saudi Arabia (S.Rehman); Department of Biosciences, Jamia Millia Islamia, New Delhi, India (Q.M.R. Haq).

E-mail addresses: ammm@edu.kau.sa, erfaan21@gmail.com (I.A. Rather), surrehman@iau.edu.sa (S. Rehman), haqqmr@gmail.com (Q.M.R. Haq).

[1] Authors contributed equally to this work.

Peer review under responsibility of King Saud University.

https://doi.org/10.1016/j.jsps.2020.10.009
1319-0164/© 2020 The Author(s). Published by Elsevier B.V. on behalf of King Saud University.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

forms (variants), exhibits increased hydrolytic activity and broader resistance to a wide range of antibiotics. β-lactam antibiotics represent the most frequently used antibiotic class in empirical therapy, however, Extended Spectrum β-Lactamases (ESBLs) capable of hydrolyzing cephalosporins and monobactams, has now evolved with co-resistance and cross-resistance to other classes of antibiotics like aminoglycosides, fluoroquinolones, rifampicin, and trimethoprim (Jacoby, 2009; Fernández-Martínez et al., 2018) and also to other anti-microbial compounds like heavy metals, biocides, disinfectants etc (Zmantar et al., 2011; Sütterlin et al., 2018, Sultan et al., 2020).

Overprescribing antibiotics or prescribing antibiotic to which bacteria has already developed resistance together with imprudent use in veterinary, aquaculture, and agriculture, are considered major factors that substantially increase the resistance burden of bacteria (Doyle et al., 2013; Ventola, 2015). Owing to the property of clustering resistance genes on single platforms like plasmids, transposons, or integrons, bacteria encoding ESBLs adopted broader resistance against a wide range of antibiotics. Rapid mobilization of resistance determinants especially under environmental conditions has exaggerated the problem, observed by a gradual shift in resistance from clinics to communities. To this rapid emergence, parameters such as pollution load, cell density, nutrient availability, temperature and pH acts as the contributing factors (Van Elsas and Bailey, 2002; Ratzke and Gore, 2018; Headd and Bradford, 2018).

Influence of the effluents from hospitals, industries, and urban settlements discharged as sewage into the aquatic ecosystem causes selection; thereby promotes the acquisition and dissemination of resistance genes (Marti et al., 2014; Rodgers et al., 2019; Fouz et al., 2020, Sabri et al., 2020). The improper disposal of unused, stored, or expired pharmaceuticals ends up in landfills, water supplies and drains that lead to contamination promotes the selection and development of resistance among inhabibting microbiota (Kraemer et al., 2019; Anwar et al., 2020). Considered as the largest tributary of river Ganga, Yamuna constitutes a major source of water for urban dwellers. Despite sharing a little (2%) catchment area across Delhi, the demand of increasing population (30.2 million according to 2020 Census) is mostly met by river water regulated at several checkpoints (Misra, 2010). The ever-increasing population and their increasing demand for potable drinking water have subjected the river to over-exploitation both quantitatively and qualitatively. Flooded with untreated and partially treated sewage, much of its pollution comes from point and non-point sources; primarily being domestic sewage, industrial discharge, the immersion of idols, cattle bathing, and agricultural runoffs (Sharma and Kansal, 2011).

Keeping a note of the situation, the present study was aimed to check the resistance pattern of bacterial isolates from the heavily polluted Delhi stretch of river Yamuna. As the river receives the discharge from domestic, hospitals, and industries with a direct impact on the selection and proliferation of resistant strains, the study was performed to (i) determine the prevalence of ESBLs producing *E. coli* and non-*E. coli* isolates and observe their susceptibility to different classes of antibiotics, and (ii) elucidate the role of abiotic factors on dissemination through the effect on conjugation rate of plasmids harboring resistance genes.

2. Methodology

2.1. Sample collection and bacterial isolation

The heavily polluted stretch of River Yamuna, traversing Delhi, the national capital territory of India was selected for the study. To observe the different discharge points of domestic sewage into the river, samples were collected from 13 different sites starting from 2.5 km upstream of Wazirabad Barrage to 6 km downstream of Okhla Barrage. Samples were collected before noon at a depth of 1–1.5 m from the river surface to avoid sunlight effect and transported under aseptic conditions for further analysis. Physical parameters like pH and temperature were recorded on spot. Avoiding fungal contamination, serially diluted samples were spread on Luria agar plates supplemented with fluconazole (20 μg/ml) and incubated overnight at 37 °C to obtain bacterial colonies. Following streaking of morphologically distinct colonies on McConky agar plates, screening for lactose fermenting *E. coli* was done by re-streaking red or pink appearing colonies on Eosin Methylin Blue (EMB) agar plates for observation of metallic sheen. Selected bacterial colonies were subjected to a preliminary biochemical (IMViC) test followed by a confirmation based on 16S rRNA gene sequencing (Jan et al., 2012; Azam et al., 2016).

(Note: To get accurate information on resistance pattern against a different class of antibiotics, isolates exhibiting similar morphology and susceptibility phenotype were considered representing a single strain).

2.2. Screening for drug susceptibility and ESBL production

A drug susceptibility test was performed using the disc diffusion method on Muller Hinton Agar (MHA) plates. Antibiotics from thirteen different categories: Aminoglycosides (amikacin, tobramycin), Carbapenems (ertapenem, imipenem), Cephamycins (cefotetan, cefoxitin), Fluoroquinolones (ciprofloxacin, levofloxacin, ofloxacin), Monobactams (aztreonam), Penicillins (ampicillin), Non-extended spectrum cephalosporins (cefuroxime, cefazolin), Extended-spectrum cephalosporins (ceftazidime, cefotaxime, ceftriaxone), Penicillin + Inhibitor (ampicillin/sulbactam), Polymyxins (polymyxin B), Rifampicins (rifampicin), Tetracyclines (tetracycline) and Trimethoprims (trimethoprim) (Himedia India), were included in the study (Magiorakos et al., 2012). Susceptibility of bacterial isolates was measured by zone inhibition test following CLSI (2019) guidelines. Multiple Antibiotic Resistance (MAR) Index referring to the resistance level against different classes of antibiotics, was calculated. MAR Index of an isolate is pondered as *a*/*b*, where *a* represents the number of antibiotics to which the isolate was resistant and *b* represents the number of antibiotics to which the isolate was subjected (Krumperman, 1983). Following the initial screen against antibiotics, bacterial isolates were screened for ESBL production by the Initial screen and Inhibitor-Potentiation Disc Diffusion (IPDD) test using MHA plates as described earlier (Azam et al., 2018). Standard strains of *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as control.

2.3. Metal tolerance test

Screened isolates were evaluated for tolerance to mercury. MIC was determined by performing broth micro-dilution assay against varied concentrations of mercuric chloride (0.2 mg/L to 10 mg/L) as reported previously (Jan et al., 2012, 2015; Azam et al., 2018). The least concentration of metal salt with no bacterial growth (OD_{600}) was considered as the MIC for the isolate.

2.4. Resistance determinant analysis of screened E. coli isolates

$ESBL^{+}$ *E. coli* isolates were screened for identification of ESBLs encoding (*bla*TEM and *bla*CTX-M) genes, along with determinants (*mer*P, *mer*T and *mer*B) imparting resistance to mercury. Total genomic DNA was extracted using PCI (phenol: chloroform: isoamyl alcohol) method from cefotaxime (2 μg/ml) supplemented overnight grown cultures in Luria-Bertani broth, with consideration to evade loss of plasmid with resistance genes during growth

under *in-vitro* conditions. Screened *E. coli* isolates were checked for the presence of resistance determinants by performing PCR using gene-specific primers described in our previous publication (Azam et al., 2016). For resistance gene localization, plasmid DNA isolated by alkaline lysis method using Qiagen Kit, was used as a template for PCR amplification. A reaction for 16S rRNA gene was run as a control to prevent any chance of genomic DNA contamination.

2.5. Conjugal transfer assay

ESBL$^+$ *E. coli* isolates showing multi-drug resistance phenotype were assessed for their conjugative R-plasmid transferability. Using three resistant strains with different resistance genes makeup (i.e. *E. coli* MRA11, *E. coli* MRB6 and *E. coli* MRE32) as a donor, conjugation experiments with *E. coli* J53 Az^R as recipient strain were performed in Luria Broth (Al-Marzooq et al., 2015).

(i) **Standard mating conditions**: Bacterial cultures in their logarithmic phase were mixed in 1:2 ratio and incubated at 37 °C for 48 hrs without shaking. Transconjugants were selected on nutrient agar plates supplemented with sodium azide (100 μg/ml) for counter-selection and cefotaxime (4 μg/ml) for plasmid-encoded resistance. Conjugation frequency was calculated as the ratio of transconjugants to donor cells. The transconjugants were assessed for susceptibility to different antibiotics and the presence of resistance genes (ESBLs and *mer* operon).

(ii) **Modified mating conditions**: To account the role of abiotic factors on plasmid transfer rate in a polluted environment, conjugation of R-plasmid was carried out at different pH, temperature, and concentrations of mercuric chloride. The effect of pH was determined by maintaining the pH of the media at 6, 6.5, 7, 7.5, 8, and 8.5. To assess the influence of temperature, the donor and recipient mixture was incubated at 25, 30, 35, 40, and 45 °C. The role of heavy metal in particular mercury on conjugation rate was evaluated by culturing donor strain in media supplemented with mercuric chloride (0.0002 mg/L, 0.002 mg/L, and 0.02 mg/L and 0.2 mg/L) for 40–50 generations and then subjected to conjugation in the presence of 0.0002 mg/L of mercuric chloride as the recipient *E. coli* J53 was intolerant to mercury concentration of 0.002 mg/L.

2.6. Biofilm formation assay

Adherence and biofilm formation ability of the test isolates was determined by crystal violet assay in 96-well microtitre plates, as described previously (O'Toole and Kolter, 1998; O'Toole, 2011). Briefly, bacterial isolates were sub-cultured in tryptic soy broth (TSB) in an automated incubator shaker at 37 °C. As the OD_{600} reached 0.5 McFarland (cell density of approximately 1.5×10^8 cfu/ml), cultures were diluted to 50% using TSB. The diluted culture (200 μl) was inoculated in a 96-well polystyrene microtiter plate and incubated at 37 °C without shaking for 48 hrs. The culture medium was aspirated and plates were gently washed four times with 200 μl of sterile phosphate buffer saline (pH 7.2) to remove the loosely associated bacteria. Cells adhered to well were stained with 200 μl of 0.1% (w/v) crystal violet at room temperature for 20 min. Following this, plates were washed with PBS (1x) to remove the excess stain. The crystal violet that stained the cells was solubilized in 200 μl of 95% (v/v) ethanol. Samples were incubated for 20 min at room temperature and biofilm formation was quantified by measuring OD_{600} with an ELISA reader (Thermo Scientific, MultiscanGo). The effect of antimicrobials at sub-MIC levels on biofilm formation was assessed by adding cefotaxime (10 mg/L) and mercuric chloride (2 mg/L) individually and in combination with the culture medium. Culture without any antimicrobial agent was used as control and wells containing TSB alone were used as blanks. The percentage of biofilm formation was calculated by the formula:

$$\text{Percentage of Biofilm formation} = ([\text{Test } OD_{600nm} - \text{Blank } OD_{600nm}]/\text{Blank } OD_{600nm}) \times 100$$

P. aerugenosa ATCC 9027 known for its biofilm formation capacity was used as a control for the study.

2.7. Statistical analysis

Statistical analysis was performed by two-sample *t*-test with the aid of GraphPad Prism software (version 8.4.3). *p*-value indicate the statistical significance of the data.

2.8. Nucleotide sequence accession numbers

Nucleotide sequences were submitted to NCBI GenBank; 16S rDNA (KC963015, KC963017-18, KC963022, KC963027-28, KJ923010-19, KJ906614-24, KJ957158-63, KM822763-69), *bla*TEM gene variants (KJ923000-09, KJ939551-60, KM593699-04, KM593706, KM873145-48) and *bla*CTX-M gene variants (KJ461948, KM873149-51, KM873153-74, KR560052).

3. Results

3.1. Sample collection and bacterial isolation

Along the 22 km long Delhi stretch of river Yamuna, water samples were collected from 13 demarked sites (Table 1) with an approximate distance of 2–2.5 kms. Getting discharged from urban settlements, sample collection was performed in April and August 2012 and 2013 to ensure maximum bacterial load. To maintain stringency and avoid unwanted redundancy of isolates, initial screening was done based on colony morphology, growth characteristic, and resistance phenotype on nutrient agar plates. A total of 227 non-duplicate bacterial isolates were isolated.

3.2. Screening for ESBL production and susceptibility pattern

Based on the results of the Initial screen and Inhibitor-Potentiation Disc Diffusion (IPDD) test, 60% (136) isolates were found ESBL producers (ESBL$^+$) and 40% (91) isolates as non-ESBL (ESBL$^-$) producers. Initial categorization of 227 non-duplicate bacterial isolates into ESBL$^+$ and ESBL$^-$ isolates are summarized in **Table S1 and S2**. Antimicrobial susceptibility tests performed for ESBL$^+$ and ESBL$^-$ isolates showed an array of susceptibility patterns towards a broad range of β-lactam and non-β-lactam classes of antibiotics (Table 2).

β-lactam: Among the ESBL$^+$ isolates, the highest resistance (71%) was observed for cefazoline, followed by cefotetan (68%), ampicillin (66%), and cefuroxime (51%). Resistant phenotype to monobactam (aztreonam) was observed among 65% isolates (Fig. 1**a & b**). In comparison to ESBL$^+$ isolates, ESBL$^-$isolates had lower resistance rates with the highest resistance observed for cefotetan (60%), followed by cefazolin (57%), ampicillin (45%), and aztreonam (44%) ($p < 0.05$). Resistance to ampicillin + sulbac tam was found among 23% ESBL$^+$ vs 9% of the ESBL$^-$ isolates ($p < 0.01$). Resistance rates to extended-spectrum cephalosporins (3rd and 4th generation) ceftazidime, cefotaxime, and ceftriaxone were observed among 81%, 70%, and 62% of the ESBL$^+$ isolates and 26%, 9%, and 4% of ESBL$^-$ isolates ($p < 0.0001$). Showing disparity in resistance to carbapenems, 64% (87) and 53% (48) of the ESBL$^+$ and ESBL$^-$ isolates were found exhibiting tolerance to ertapenem ($p < 0.05$), while only 16 (12%) and 8 (9%) of ESBL$^+$ and ESBL$^-$ isolates were resistant to imipenem tested.

Table 1
Description of sample collection sites of Delhi stretch of river Yamuna, India.

Site Code	Name of the Drain	Location of Sampling site	Longitude	Latitude	Temp. (°C)	pH
SA	–	2.5 km upstream of Wazirabad Barrage	77°14′09.22″E	28°43′39.26″N	22	8.64 ± 0.05
SB	Najafgarh Drain	Wazirabad Barrage	77°13′52.83″E	28°42′23.36″N	19	7.58 ± 0.05
SC	Sweeper colony Drain	2.5 km downstream of wazirabad Barrage	77°13′45.77″E	28°41′38.09″N	23	7.28 ± 0.05
SD	Metcalf House Drain	ISBT Pul	77°14′05.36″E	28°40′07.89″N	22	7.64 ± 0.05
SE	Civil Mill Drain	Old Railway bridge	77°14′51.42″E	28°39′41.77″N	21	7.42 ± 0.05
SF	Delhi Gate Drain	2.5 km downstream of railway bridge	77°15′50.44″E	28°39′01.06″N	28	7.27 ± 0.05
SG	Sen Nursing Home Drain	Yamuna Barrage	77°15′13.34″E	28°37′37.56″N	24	7.14 ± 0.05
SH	Drain No-14A	Railway track (overbridge)	77°15′19.15″E	28°36′47.95″N	25	7.55 ± 0.05
SJ	–	Kanal Colony	77°17′43.08″E	28°34′01.43″N	24	7.43 ± 0.05
SK	LPG Bottling Plant Drain	Okhla Barrage	77°19′01.38″E	28°32′34.21″N	23	7.27 ± 0.05
SL	Shahadara Drain	1.25Km downstream of Okhla Barrage	77°19′24.80″E	28°32′24.67″N	25	7.38 ± 0.05
SM	–	6Km downstream of Okhla Barrage	77°20′46.14″E	28°30′38.97″N	27	7.31 ± 0.05

Table 2
Non-susceptibility to antibiotics of different classes among ESBL+ and ESBL− isolates.

	Total no. of non-duplicate bacteria isolated (n = 227)		
	ESBL phenotype	ESBL+	ESBL-
	Isolates with ESBL phenotype	60% (n = 136)	40% (n = 91)
Antibiotic category	Antibiotic name	Resistance phenotype[a] among ESBL$^+$ (%)	Resistance phenotype[a] among ESBL$^-$ (%)
β-lactam	Ampicillin	74	57
	Ampicillin/ Sulbactam	38	15
	Aztreonam	78	52
	Cefotetan	76	70
	Cefoxitin	52	40
	Cefuroxime	65	44
	Cefazolin	78	76
	Ceftazidime	94	44
	Cefotaxime	90	33
	Ceftriaxone	86	11
	Ertapenem	80	59
	Imipenem	27	21
Non-β-lactam	Ciprofloxacin	86	76
	Levofloxacin	26	13
	Ofloxacin	23	16
	Plymixin B	52	52
	Rifampicin	77	79
	Tetracycline	20	12
	Amikacin	35	37
	Tobramycin	32	27
	Trimethoprim	32	16

[a] Isolates with intermediate susceptible phenotype as per CLSI standards were included in the resistant phenotype category. In Fig. 1, intermediate category is shown separately.

***Non-β-lactam*:** Maximum resistance to non-β-lactam classes of antibiotics was observed for rifampicin, with 62% of ESBL$^+$ and 67% of ESBL$^-$ isolates (Fig. 1**c & d**). Resistance to ciprofloxacin was among 44% (60/136) of ESBL$^+$ and 27% (24/91) of ESBL$^-$ isolates ($p < 0.01$). On one side, where polymixin B resistance was among nearly half (52%) of ESBL$^+$ and ESBL$^-$ isolates, the least resistance was observed for tetracycline and ofloxacin, to which nearly 10% of ESBL$^+$ and 5% ESBL$^-$ isolates were found resistant. The non-susceptibility of isolates towards levofloxacin and ofloxacin was observed for 16% and 10% of ESBL$^+$ and 8% and 5% of ESBL$^-$ isolates, respectively. Compared to other non-β-lactam classes of antibiotics, resistance to aminoglycoside class of antibiotic was found higher among ESBL$^-$ with 26% of isolates showing resistance to amikacin and tobramycin, followed by ESBL$^+$ isolates that exhibit only 18% ($p < 0.05$) and 22% resistance to amikacin and tobramycin, respectively.

In lieu of resistant phenotype to at least 10 of the 21 antibiotics tested, the MAR index value of ≥0.5 was observed for 68% (92) of ESBL$^+$ and 24% (22) of ESBL$^-$ isolates. The detailed information regarding the susceptibilities of isolates to different antibiotics is summarized in **Table S3-S6**. Among 136 ESBL$^+$ isolates, 78.6% (107) had MDR, 16.9% (23) XDR, and 0.7% (1) PDR phenotype; with only one isolate that was sensitive to nearly all antibiotics except the cephalosporins. Of the 91 ESBL$^-$ isolates, 90% (82) had MDR and 3%(3) XDR phenotype; with no isolate having the PDR phenotype. Contrarily, 3 isolates of the ESBL$^-$ category were found sensitive to all the antibiotics tested. This represents that proportion of isolates with the XDR phenotype is significantly higher in the case of ESBL$^+$ compared to that of ESBL$^-$ isolates.

Among 136 ESBL$^+$ isolates, the MIC value for mercuric chloride was observed as 10 mg/L in 38 isolates, 4 mg/L in 93 isolates, and 2 mg/L among 4 isolates. But only 20% (18) of ESBL$^-$ isolates were found having MIC value of 10 mg/L, 59 isolates 4 mg/L, and 13 isolates 2 mg/L of the mercuric chloride (**Table S7 & S8**).

3.3. Screening for E. coli and determination of resistance genes

The sequence analysis of 16S rRNA gene revealed 25% (34) isolates of ESBL$^+$ and 36% (35) of ESBL$^-$ isolates as *E. coli*. Among the ESBL$^+$ *E. coli* isolates 76% (26) isolates harbored the *bla*TEM gene, 71% (24) *bla*CTX-M and 56% (19) had both *bla*TEM and *bla*CTX-M genes. The mercury transport genes (*mer*P and *mer*T) imparting resistances were present in 68% (23) and 74% (25) isolates, respectively, and *mer*B encoding organomercurial lyase catalyzing proteolytic cleavage of Hg-C bond was present among 50% (17) isolates.

3.4. Conjugation assay

The ESBL$^+$ *E. coli* isolates were screened for the presence of resistance genes on plasmid DNA and subsequently, three isolates i.e. *E. coli* MRA11, *E. coli* MRB6, and *E. coli* MRE32, were subjected to conjugation studies. Sequence analysis of an amplified product revealed *E. coli* MRA11 harboring *bla*TEM-1 and *bla*TEM-116, *E. coli* MRB6 *bla*TEM-116, and *bla*CTX-M-71 and *E. coli* MRE32 having *bla*TEM-116, *bla*CTX-M-3, and *bla*CTX-M-15 determinants, respectively. Association of mercury resistance determinants (*mer*B, *mer*P, and *mer*T genes) on the plasmid were found among all three isolates. The detailed information regarding the localization of resistance genes of donor and trans-conjugates is summarized in Table 3.

The effect of pH change on the conjugation rate was tested for pH 6–8.5 range and maximum conjugation frequency was observed at pH 7 for *E. coli* MRA11 (4.7×10^{-5}), *E. coli* MRB6 (2.4×10^{-5}) and *E. coli* MRE32 ($3.77x\ 10^{-4}$). A higher conjugation rate was observed near-neutral pH (pH 7–7.5) (Fig. 2**a**). An increase or a decrease in pH of the culture medium showed a decrease in

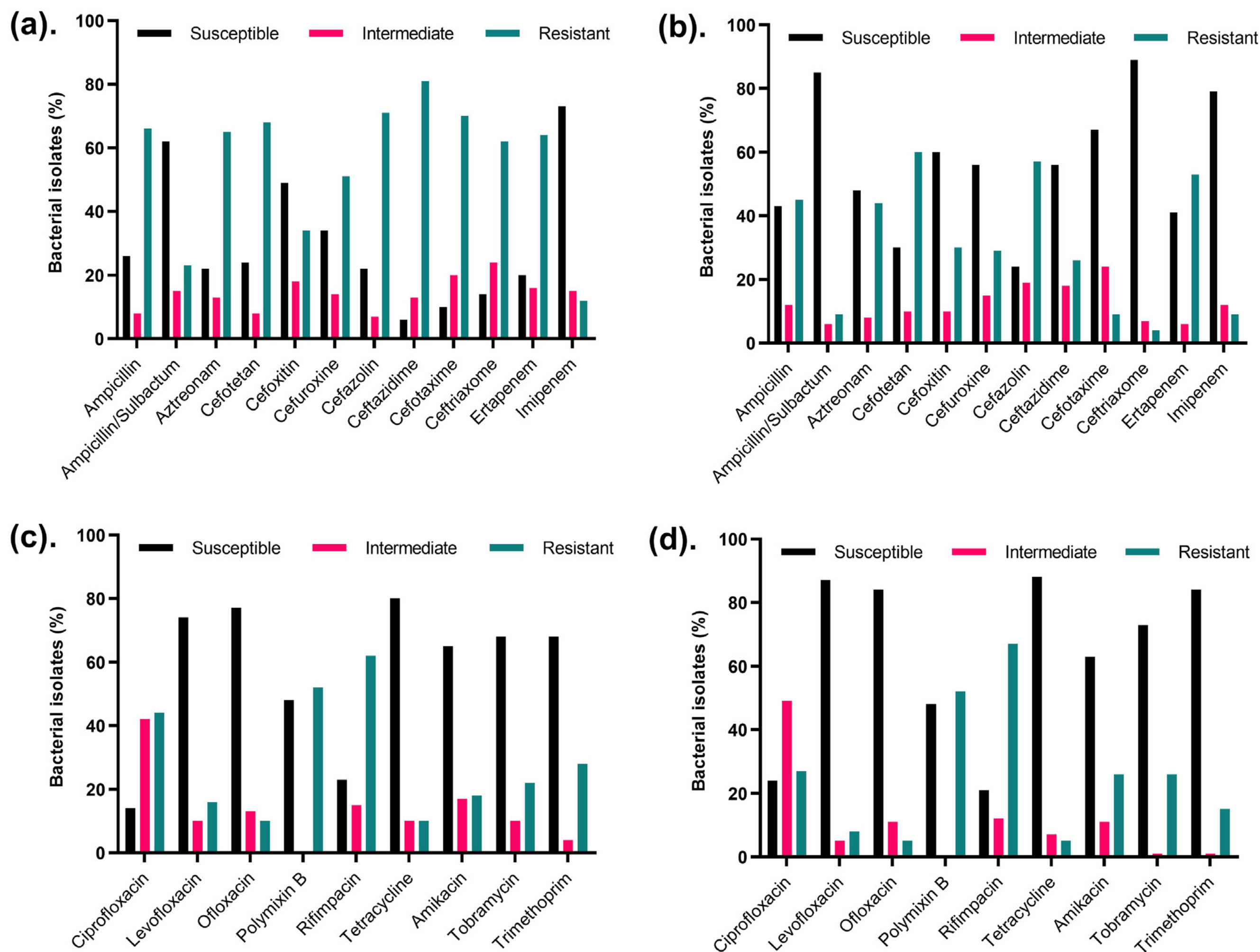

Fig. 1. Susceptibility pattern of (a) ESBL producers towards β-lactam class of antibiotics (b) non-ESBL producers towards β-lactam class of antibiotics (c) ESBL producers towards non-β-lactam class of antibiotics (d) non-ESBL producers towards non-β-lactam class of antibiotics. Ampicillin (10 μg), Ampicillin/sulbactam (10/10 μg), Aztreonam (30 μg), Cefotetan (30 μg), Cefoxitin (30 μg), Cefuroxime (30 μg), Cefazolin (10 μg), Ceftazidime (30 μg), Cefotaxime (30 μg), Ceftriaxone (30 μg), Ertapenem (10 μg), Imipenem (10 μg), Ciprofloxacin (5 μg), Levofloxacin (5 μg), Ofloxacin (5 μg), Polymixin B (300 units), Rifampicin (5 μg), Tetracyclin (30 μg), Amikacin (30 μg), Tobramycin (10 μg), and Trimethoprim (5 μg).

Table 3
ESBL and *mer* operon genes localized on chromosomal and plasmid DNA among ESBL$^+$ *E. coli* isolates.

ESBL positive *E. coli* Isolate	Size of pDNA	Genes amplified from total DNA		Genes amplified from pDNA	
		ESBL gene(s)	*mer* operon gene(s)	ESBL gene(s)	*mer* operon gene(s)
E. coli MRA11	>21	TEM-1, TEM-116	*mer*P, *mer*T, *mer*B	TEM-116	*mer*P, *mer*T, *mer*B
E. coli MRB6	~20	TEM-116, CTX-M-71	*mer*P, *mer*T, *mer*B	TEM-116, CTX-M-71	*mer*P, *mer*T
E. coli MRE32	~20	TEM-116, CTX-M-3, CTX-M-15	*mer*P, *mer*T, *mer*B	TEM-116, CTX-M-15	*mer*P, *mer*T, *mer*B

the conjugation rate as observed by the decrease in transconjugant numbers.

With the change in mating temperature, maximum conjugation frequency was observed at 40 °C for *E. coli* MRB6 (4.1×10^{-5}) and *E. coli* MRE32 (4.89×10^{-4}) and 35 °C for the isolate *E. coli* MRA11. The transfer rate showed an increasing trend with an increase in temperature from 20 °C to 45 °C, reaching its maximum value at 40 °C and then declined at 45 °C. The results corresponding to the effect of mating temperature on the conjugation rate are shown in Fig. 2**b.**

For the effect of heavy metal on conjugation rate, maximum numbers of transconjugants were obtained at 0.002 mg/L of mercuric chloride that represents 10^3 times lower concentration than MIC values of *E. coli* MRA11 and *E. coli* MRE32. However, an overall decline in the conjugation rate was observed on the addition of mercury chloride (as low as 0.0002 mg/L) to the mating medium. In absence of metal salt, conjugation frequency was reported to be 4.5×10^{-5}, 3.0×10^{-5} and 4.14×10^{-4} for *E. coli* MRA11, *E. coli* MRB6, and *E. coli* MRE32 isolates, respectively. On addition of 0.0002 mg/L of mercuric chloride to the culture medium, the conjugation frequency declined to 2.81×10^{-5}, 1.74×10^{-5} and 2.88×10^{-4} corresponding to *E. coli* MRA11, *E. coli* MRB6, and *E. coli* MRE32 isolates, respectively (Fig. 2**c**).

3.5. Biofilm formation

The control culture of *P. aeruginosa* ATCC 9027 showed a 20.4% biofilm formation in TSB. However, the biofilm formation observed

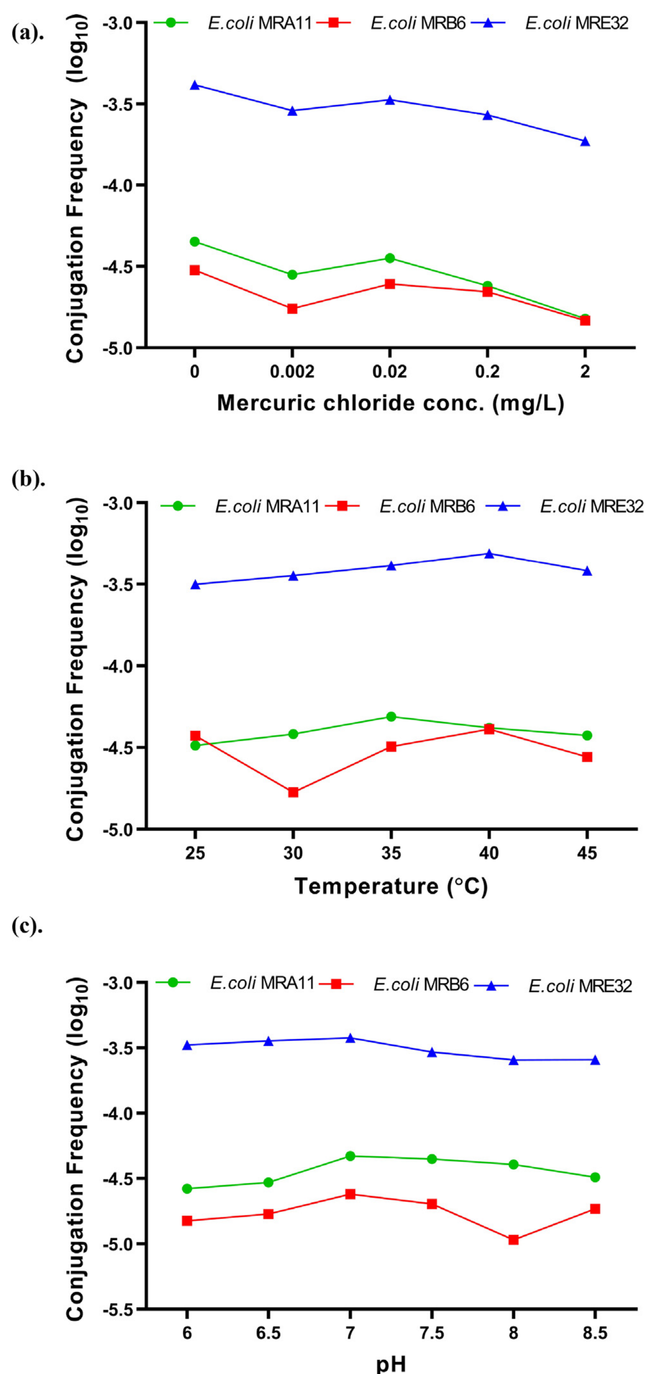

Fig. 2. Conjugation frequency of ESBL producing *E. coli* isolates under different biophysical parameters. (a) Conjugation rate at different pH of media, (b) Conjugation rate at different incubation temperatures, (c) Conjugation rate of *E. coli* grown under varied concentrations of mercuric chloride. (—●—) *E. coli* MRA11, (—■—) *E. coli* MRB6; (—▲—) *E. coli* MRE32.

a 22.4% increment on supplementing media with cefotaxime (10 μg/ml) and an increase of 23% on the addition of mercuric chloride (2 mg/L). Their combination (cefotaxime and mercuric chloride) to the medium showed a 33.4% increase in biofilm formation. Summarized results of the biofilm formation rate under different media conditions are presented in Fig. 3. Biofilm formation was found to be 29.6% for *E. coli* MRA11 and *E. coli* MRB6; and 24.6% in *E. coli* MRE32. A decrease of 17.6%, 3.6%, and 4.2% in biofilm formation was observed on the addition of cefotaxime alone at zero hrs for *E. coli* MRA11, *E. coli* MRB6, and *E. coli* MRE32, respectively. Surprisingly, when media was supplemented with mercuric chloride the biofilm formation was increased i.e.59.5% in *E. coli* MRA11, 90.2% in the case of *E. coli* MRB6, and 49.6% in *E. coli* MRE32. When cefotaxime and mercuric chloride were used in combination, biofilm formation was 29.6%, 43.4%, and 30.2% for the isolates *E. coli* MRA11, *E. coli* MRB6, and *E. coli* MRE32, representing a decrease than what was observed for media supplemented with metal alone.

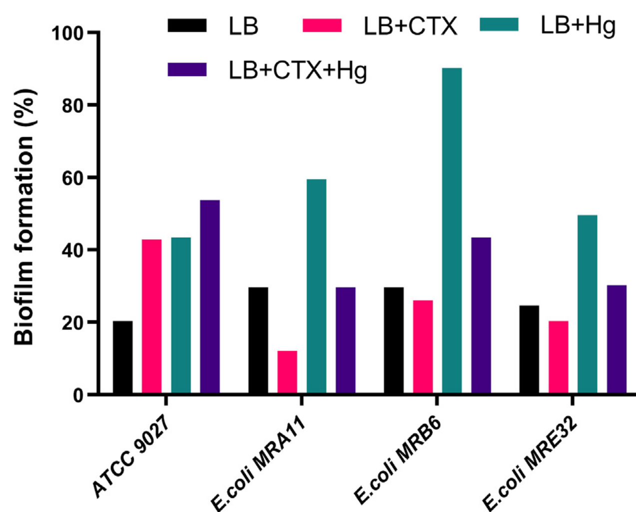

Fig. 3. Biofilm formation in test ESBL positive *E. coli* isolates under antimicrobial stressed conditions. (■) LB only; (■) LB supplemented with cefotaxime (10 mg/L); (■) LB supplemented with mercuric chloride (2 mg/L); (■) LB supplemented with cefotaxime (10 mg/L) and mercuric chloride (2 mg/L).

4. Discussion

Recounting potential threat for human health in terms of maintenance of resistance gene pool and their movement across diverse groups of bacteria in the urban aquatic environment, it seems imperative to have an understanding of the resistance profile of inhabitant bacteria that could contribute to infections among the human host. In the present study, we have described the prevalence rate of ESBL producing and non-ESBL producing bacteria in anthropogenically influenced river water from the capital city of India. Moreover, the effect of physiological factors like pH, temperature, and heavy metal on the transmission pattern of resistance genes was studied.

The present study revealed a high prevalence of ESBL producing bacteria in Delhi stretch of river Yamuna. The increased incidence of $ESBL^+$ *E. coli* isolates in the aquatic environment are in concordance with the study of Taneja et al. (2010) conducted in the same region on nosocomial blood samples in GB Pant Hospital, Delhi that report 41.7% of *E. coli* and 70.7% of *K. pneumonia* as ESBL producers. In other similar studies conducted on samples from NIMS hospital, Rajasthan, and VMKV hospital, Tamil Nadu, India; Sharma et al. (2013) and Rao et al. (2015) reported 52.49% and 62% isolates having an $ESBL^+$ phenotype.

A multi-drug resistance phenotype was observed among both $ESBL^+$ and $ESBL^-$ isolates, however, the resistance profile was higher for $ESBL^+$ isolates (Table 2). The increased resistance to multiple class of antibiotics among $ESBL^+$ isolates was also observed among other studies showing a sharp increase in resistance among both clinical and community environments (Pal et al., 2015; Yadav et al., 2015; Gonzalez-Villoria and Valverde-Garduno, 2016; Laxminarayan and Chaudhury, 2016). Co-resistance to a non-β-lactam class of antibiotics (aminoglycosides, fluoroquinolones,

polymixins, and trimethoprims) observed for both ESBL+ and ESBL− isolates were similar to epidemiological studies suggesting co-selection of multiple resistance genes. Increased resistance to the aminoglycoside class of antibiotics (amikacin and tobramycin) observed in the study could be attributed to reduced uptake, decrease in permeability, alterations in the ribosomal binding sites, or by the production of aminoglycoside-modifying enzymes (Mingeot-Leclercq et al., 1999; Ramirez and Tolmasky, 2010). High resistance to aminoglycosides, quinolones, and tetracyclines and their mobilization through conjugative plasmids was also prevalent in Portugal as reported by Tacão et al. (2012, 2014).

Besides, several groups report the co-organization of genes in a cassette that confers resistance against a variety of contaminants as well as antibiotics (McArthur and Tuckfield, 2000; Stepanauskas et al., 2006; McIntosh et al., 2008; Pal et al., 2015; Thomas et al., 2020). The presence of antibiotics and/or metals even at far below the inhibitory concentration is considered as a driving force favoring a better adaptation of bacteria. In a polluted environment, bacterial isolates were able to tolerate different concentrations of mercury that suggest the operation of different modes of detoxification encoded by genes located on operons. As an adaptation to bacteria, mercury resistance is not only correlated with the ability to live in mercury-containing environments but also to survive other forms of toxicity as well. In this regard, *mer* operon genes were frequently observed to be genetically linked to antibiotic resistance genes (Mirzaei et al., 2013; Li et al., 2017; Lloyd et al., 2018).

Highlighting the importance of the polluted environment in the dissemination of resistance genes, the study of abiotic factors on resistance gene transfer and biofilm formation among ESBL producing bacteria was performed. Playing an important role in the adaptation of bacterial species to the changing environment, the effect of pH in the neutral range (pH 7–8) was found to favor the dissemination of resistance plasmids. Though no significant effect of pH change was observed in the studies of Fernandez-Astorga et al. (1992) and Al-Masaudi et al. (1991), extremes of both acidic and alkaline conditions were found hampering the transfer frequency for all three isolates in the study. An increase in conjugation rate with an increase in temperature from 20 °C to 45 °C was observed, which was found to be in concordance with previous studies (Al-Masaudi et al., 1991; Fernandez-Astorga et al., 1992; Headd and Bradford, 2018). With the maximum number of transconjugants obtained in the temperature range of 35–40 °C, a substantial decrease was observed at 45 °C. The observed change could be the result of a change in cell physiology. Compared to control (media alone), the addition of mercuric chloride to the culture medium reduced the conjugation rate in all the three isolates. Of the different concentrations of mercuric chloride tested, the maximum number of transconjugants were obtained at 0.002 mg/L as shown in Fig. 2. It is hypothesized that the presence of mercuric chloride at sub-MIC levels promptly increases the R-plasmid copy number for the increased fitness of isolates in the stressed environment. Moreover, metal induces oxidative stress, bacterial cell membrane damage, enhances the expression of mating pair formation genes and DNA transfer, and replication genes (Qiu et al., 2012). Further increase in metal concentration generates pronounced metal stress and as such overcasts the fitness cost.

The change in biofilm formation under the differential composition of growth medium and changing environmental conditions are well documented (Bjergbæk et al., 2006; Marsden et al., 2017). Here, we tried to figure out the impact of antimicrobial agents (cefotaxime, mercuric chloride, and their combination) on the biofilm formation rate of multidrug-resistant ESBL+ *E. coli* isolates. Maximum biofilm formation was found on culturing isolates in the medium supplemented with mercuric chloride and least on addition of cefotaxime (Fig. 3). The mechanism underlying differential biofilm formation under varied conditions needs further exploration, however, increased biofilm formation under the sub-inhibitory concentration of cadmium and nickel has been documented by Wu et al. (2015) and Perrin et al. (2009). Currently, the increased biofilm formation in media supplemented with a sub-inhibitory concentration of antimicrobial agent is hypothesized to be due to increased expression of *rpoS*, reported to be associated with an enhancement in biofilm formation, stress response induction (Ito et al., 2009), and also in attributing multidrug resistance (Rami et al., 2005). In addition, to decrease the susceptibility to antimicrobials, biofilm formation increases the dissemination of resistance genes through enhancement in the recombination events between phylogenetically related and distant bacteria as part of adaptation to the changing environment (Gebreyohannes et al., 2019).

With poor sanitary conditions and increased discharge of antimicrobial compounds, acquisition of resistance among natural inhabitants of natural water bodies not only increase the resistance burden but also facilitate the transfer of resistance genes to human commensal and pathogenic bacteria. Present data will substantially help to frame policies and measures for affirmative action to prevent the selection and spread of multidrug resistance. The emergence of the pan- and extensively drug-resistant bacterial strains in such an aquatic environment of urban area foretells a dark phase where management of infection seems to be of utmost importance, and where the scene has been horrified by the unavailability of new drugs. With this, advanced drug resistance surveillance and molecular characterization of ESBL producing isolates are necessary to guide the appropriate and judicious antibiotic use. A better understanding of resistance profile and mechanisms that contribute to the emergence and spread of resistance holds great essentiality in the infection control programs.

Funding

The project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant No. (DF-702-130-1441). The authors, therefore, gratefully acknowledge DSR technical and financial support.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2020.10.009.

References

Al-Marzooq, F., Yusof, M.Y.M., Tay, S.T., 2015. Molecular analysis of antibiotic resistance determinants and plasmids in Malaysian isolates of multidrug resistant *Klebsiella pneumoniae*. PloS One 10 (7), e0133654. https://doi.org/10.1371/journal.pone.0133654.

Al-Masaudi, S.B., Day, M.J., Russell, A.D., 1991. Antibiotic resistance and gene transfer in *Staphylococcus aureus*. J. Appl. Bacteriol. 70, 279–290. https://doi.org/10.1111/j.1365-2672.1991.tb02937.x.

Anwar, M., Iqbal, Q., Saleem, F., 2020. Improper disposal of unused antibiotics: an often overlooked driver of antimicrobial resistance. Expert Rev. Anti. Infect. Ther. 1–3. https://doi.org/10.1080/14787210.2020.1754797.

Azam, M., Jan, A.T., Haq, Q.M., 2016. *bla*CTX-M-152, a novel variant of CTX-M-group-25, identified in a study performed on the prevalence of multidrug resistance among natural inhabitants of river Yamuna, India. Front. Microbiol. 7, 176. https://doi.org/10.3389/fmicb.2016.00176.

Azam, M., Jan, A.T., Kumar, A., Siddiqui, K., Mondal, A.H., Haq, Q.M., 2018. Study of pandrug and heavy metal resistance among *E. coli* from anthropogenically

influenced Delhi stretch of river Yamuna. Braz. J. Microbiol. 49 (3), 471–480. https://doi.org/10.1016/j.bjm.2017.11.001.
Bassetti, M., Peghin, M., Vena, A., Giacobbe, D.R., 2019. Treatment of infections due to MDR Gram-negative bacteria. Front. Med. 6, 74.
Bjergbæk, L.A., Haagensen, J.A.J., Reisner, A., Molin, S., Roslev, P., 2006. Effect of oxygen and growth medium on in vitro biofilm formation by *Escherichia coli*. Biofilms 3 (1), 1.
Davey, M.S., Tyrrell, J.M., Howe, R.A., Walsh, T.R., Moser, B., Toleman, M.A., Eberl, M., 2011. A promising target for treatment of multidrug-resistant bacterial infections. Antimicrob. Agent. Chemother. 55 (7), 3635–3636.
Doyle, M.P., Loneragan, G.H., Scott, H.M., Singer, R.S., 2013. Antimicrobial resistance: challenges and perspectives. Comp. Rev. Food Sci. Food Saf. 12 (2), 234–248. https://doi.org/10.1111/1541-4337.12008.
Fernandez-Astorga, A.U.R.O.R.A., Muela, A.L.I.C.I.A., Cisterna, R.A.M.O.N., Iriberri, J.U. A.N., Barcina, I.S.A.B.E.L., 1992. Biotic and abiotic factors affecting plasmid transfer in *Escherichia coli* strains. App. Environ. Microbiol. 58 (1), 392–398.
Fernández-Martínez, M., Ruiz del Castillo, B., Lecea-Cuello, M.J., Rodríguez-Baño, J., Pascual, Á., Martínez-Martínez, L., Spanish Network for the Research in Infectious Diseases (REIPI), Spanish Group for Nosocomial Infections (GEIH), 2018. Prevalence of aminoglycoside-modifying enzymes in *Escherichia coli* and *Klebsiella pneumoniae* producing extended spectrum β-lactamases collected in two multicenter studies in Spain. Microb. Drug Res. 24 (4), 367–376.
Fouz, N., Pangesti, K.N., Yasir, M., Al-Malki, A.L., Azhar, E.I., Hill-Cawthorne, G.A., Ghany, M.A.E., 2020. The contribution of wastewater to the transmission of antimicrobial resistance in the environment: implications of mass gathering settings. Trop. Med. Infect. Dis. 5 (1), 33. https://doi.org/10.3390/tropicalmed5010033.
Gebreyohannes, G., Nyerere, A., Bii, C., Sbhatu, D.B., 2019. Challenges of intervention, treatment, and antibiotic resistance of biofilm-forming microorganisms. Heliyon. 5 (8), e02192. https://doi.org/10.1016/j.heliyon.2019.e02192.
Gonzalez-Villoria, A.M., Valverde-Garduno, V., 2016. Antibiotic-resistant *Acinetobacter baumannii* increasing success remains a challenge as a nosocomial pathogen. J. Pathog. 1–10. https://doi.org/10.1155/2016/7318075.
Headd, B., Bradford, S.A., 2018. Physicochemical factors that favor conjugation of an antibiotic resistant plasmid in non-growing bacterial cultures in the absence and presence of antibiotics. Front. Microbiol. 9, 2122. https://doi.org/10.3389/fmicb.2018.02122.
Ito, A., Taniuchi, A., May, T., Kawata, K., Okabe, S., 2009. Increased antibiotic resistance of *Escherichia coli* in mature biofilms. App. Environ. Microbiol. 75 (12), 4093–4100. https://doi.org/10.1128/AEM.02949-08.
Jacoby, G.A., 2009. AmpC β-lactamases. Clin. Microbiol. Rev. 22 (1), 161–182. https://doi.org/10.1128/CMR.00036-08.
Van Elsas, J.D., Bailey, M.J., 2002. The ecology of transfer of mobile genetic elements. FEMS Microbiol. Ecol. 42 (2), 187–197. https://doi.org/10.1111/j.1574-6941.2002.tb01008.x.
Jan, A.T., Azam, M., Ali, A., Choi, I., Haq, Q.M.R., 2015. Analysis for the presence of determinants imparting resistance to mercury among bacterial inhabitants of polluted water bodies. Braz. J. Microbiol. 47, 55–62.
Jan, A.T., Azam, M., Ali, A., Haq, Q.M.R., 2012. Molecular characterization of mercury resistant bacteria inhabiting polluted water bodies of different geographical locations in India. Curr. Microbiol. 65 (1), 14–21. https://doi.org/10.1007/s00284-012-0118-3.
Kraemer, S.A., Ramachandran, A., Perron, G.G., 2019. Antibiotic pollution in the environment: from microbial ecology to public policy. Microorganisms. 7 (6), 180. https://doi.org/10.3390/microorganisms7060180.
Krumperman, P.H., 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. App. Environ. Microbiol. 46 (1), 165–170.
Laxminarayan, R., Chaudhury, R.R., 2016. Antibiotic resistance in India: drivers and opportunities for action. PLoS Med. 13 (3), e1001974. https://doi.org/10.1371/journal.pmed.1001974.
Li, L.G., Xia, Y., Zhang, T., 2017. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. ISME J. 11 (3), 651–662. https://doi.org/10.1038/ismej.2016.155.
Lloyd, N.A., Nazaret, S., Barkay, T., 2018. Whole genome sequences to assess the link between antibiotic and metal resistance in three coastal marine bacteria isolated from the mummichog gastrointestinal tract. Mar. Poll. Bull. 135, 514–520.
Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., et al., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infec. 18 (3), 268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x.
Marsden, A.E., Grudzinski, K., Ondrey, J.M., DeLoney-Marino, C.R., Visick, K.L., 2017. Impact of salt and nutrient content on biofilm formation by Vibrio fischeri. PLoSOne 12 (1), e0169521.
Marti, E., Variatza, E., Balcazar, J.L., 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trend Microbiol. 22 (1), 36–41. https://doi.org/10.1016/j.tim.2013.11.001.
McArthur, J.V., Tuckfield, R.C., 2000. Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. App. Environ. Microbiol. 66 (9), 3722–3726. https://doi.org/10.1128/AEM.66.9.3722-3726.2000.
McIntosh, D., Cunningham, M., Ji, B., Fekete, F.A., Parry, E.M., Clark, S.E., et al., 2008. Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the Salmonella enterica plasmid pSN254. J. Antimicrob. Chemother. 61 (6), 1221–1228. https://doi.org/10.1093/jac/dkn123.
Mingeot-Leclercq, M.P., Glupczynski, Y., Tulkens, P.M., 1999. Aminoglycosides: activity and Resistance. Antimicrob. Agent. Chemother. 43 (4), 727–737.
Mirzaei, N., Rastegari, H., Kargar, M., 2013. Antibiotic resistance pattern among gram negative mercury resistant bacteria isolated from contaminated environments. Jundishapur J. Microbiol. 6 (10), e8085. https://doi.org/10.5812/jjm.8085.
Misra, A.K., 2010. A river about to die: Yamuna. J. Water Res. Prot. 2, 489–500. https://doi.org/10.4236/jwarp.2010.25056.
O'Toole, G.A., 2011. Microtiter dish biofilm formation assay. JoVE J. Vis. (Exp) 47, e2437. https://doi.org/10.3791/2437.
O'Toole, G.A., Kolter, R., 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. 28 (3), 449–461.
Pal, C., Bengtsson-Palme, J., Kristiansson, E., Larsson, D.J., 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genomics 16 (1), 964. https://doi.org/10.1186/s12864-015-2153-5.
Perrin, C., Briandet, R., Jubelin, G., Lejeune, P., Mandrand-Berthelot, M.A., Rodrigue, A., et al., 2009. Nickel promotes biofilm formation by *Escherichia coli* K-12 strains that produce curli. App. Environ. Microbiol. 75 (6), 1723–1733. https://doi.org/10.1128/AEM.02171-08.
Qiu, Z., Yu, Y., Chen, Z., Jin, M., Yang, D., Zhao, Z., et al., 2012. Nanoalumina promotes the horizontal transfer of multiresistance genes mediated by plasmids across genera. Proc. Natl. Acad. Sci. USA 109 (13), 4944–4949.
Rami, A., Toutain, C.M., Jacq, A., 2005. An increased level of alternative sigma factor RpoS partially suppresses drug hypersensitivity associated with inactivation of the multidrug resistance pump AcrAB in *Escherichia coli*. Res. Microbiol. 156 (3), 356–360. https://doi.org/10.1016/j.resmic.2004.10.015.
Ramirez, M.S., Tolmasky, M.E., 2010. Aminoglycoside modifying enzymes. Drug Resist. Updates 13 (6), 151–171. https://doi.org/10.1016/j.drup.2010.08.003.
Ratzke, C., Gore, J., 2018. Modifying and reacting to the environmental pH can drive bacterial interactions. PLoS Biol. 16 (3), e2004248. https://doi.org/10.1371/journal.pbio.2004248.
Rao, A.V.R., Seetha, S., Rajan, R., 2015. Prevalence of extended spectrum β-lactamases among gram negative clinical isolates from a tertiary care hospital. Int. J. Pharm. Bio. Sci. 6 (3), 1295–1304.
Rodgers, K., McLellan, I., Peshkur, T., Williams, R., Tonner, R., Hursthouse, A.S., et al., 2019. Can the legacy of industrial pollution influence antimicrobial resistance in estuarine sediments. Environ. Chem. Lett. 17 (2), 595–607.
Sabri, N.A., Schmitt, H., Van der Zaan, B., Gerritsen, H.W., Zuidema, T., Rijnaarts, H.H. M., et al., 2020. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. J. Environ. Chem. Eng. 8 (1), 102245.
Sharma, D., Kansal, A., 2011. Water quality analysis of River Yamuna using water quality index in the national capital territory, India (2000–2009). App. Water Sci. 1 (3–4), 147–157. https://doi.org/10.1007/s13201-011-0011-4.
Sharma, M., Pathak, S., Srivastava, P., 2013. Prevalence and antibiogram of Extended Spectrum β-Lactamase (ESBL) producing Gram negative bacilli and further molecular characterization of ESBL producing *Escherichia coli* and *Klebsiella spp*. J. Clin. Diagn. Res. 7 (10), 2173–2177. https://doi.org/10.7860/JCDR/2013/6460.3462.
Stepanauskas, R., Glenn, T.C., Jagoe, C.H., Tuckfield, R.C., Lindell, A.H., King, C.J., et al., 2006. Co-selection for microbial resistance to metals and antibiotics in freshwater microcosms. Environ. Microbiol. 8 (9), 1510–1514. https://doi.org/10.1111/j.1462-2920.2006.01091.x.
Sultan, I., Ali, A., Gogry, F.A., Rather, I.A., Sabir, J.S., Haq, Q.M.R., 2020. Bacterial isolates harboring antibiotics and heavy-metal resistance genes co-existing with mobile genetic elements in natural aquatic water bodies. Saudi J. Biol. Sci., 1–9
Sütterlin, S., Téllez-Castillo, C.J., Anselem, L., Yin, H., Bray, J.E., Maiden, M.C., 2018. Heavy metal susceptibility of *Escherichia coli* isolated from urine samples from Sweden, Germany, and Spain. Antimicrob. Agent. Chemother. 62 (5), e00209-18. https://doi.org/10.1128/AAC.00209-18.
Tacão, M., Correia, A., Henriques, I., 2012. Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of *bla* CTX-M-like genes. Appl. Environ. Microbiol. 78 (12), 4134–4140. https://doi.org/10.1128/AEM.00359-12.
Tacão, M., Moura, A., Correia, A., Henriques, I., 2014. Co-resistance to different classes of antibiotics among ESBL-producers from aquatic systems. Water Res. 48, 100–107.
Taneja, J., Mishra, B., Thakur, A., Dogra, V., Loomba, P., 2010. Nosocomial blood-stream infections from extended-spectrum-beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumonia* from GB Pant Hospital, New Delhi. J. Infect. Dev. Countr. 4 (08), 517–520. https://doi.org/10.3855/jidc.668.
Thomas IV, J.C., Oladeinde, A., Kieran, T.J., Finger Jr, J.W., Bayona-Vásquez, N.J., Cartee, J.C., et al., 2020. Co-occurrence of antibiotic, biocide, and heavy metal resistance genes in bacteria from metal and radionuclide contaminated soils at the Savannah River Site. Microb. Biotechnol. 13, 1179–1200.
Ventola, C.L., 2015. The antibiotic resistance crisis: part 1: causes and threats. Pharm. Therapeu. 40 (4), 277–283.
Yadav, K.K., Adhikari, N., Khadka, R., Pant, A.D., Shah, B., 2015. Multidrug resistant Enterobacteriaceae and extended spectrum β-lactamase producing *Escherichia*

coli: a cross-sectional study in National Kidney Center, Nepal. Antimicrob. Resist. Infect. Cont. 4 (1), 42. https://doi.org/10.1186/s13756-015-0085-0.

Wu, X., Santos, R.R., Fink-Gremmels, J., 2015. Cadmium modulates biofilm formation by Staphylococcus epidermidis. Int. J. Env. Res. Pub. Health. 12 (3), 2878–2894.

Zmantar, T., Kouidhi, B., Miladi, H., Bakhrouf, A., 2011. Detection of macrolide and disinfectant resistance genes in clinical Staphylococcus aureus and coagulase-negative staphylococci. BMC Res Notes. 4 (453), 1–9. https://doi.org/10.1186/1756-0500-4-453.