Report of antibiotic resistance in urban and rural wastewaters from West Bengal, India

ABSTRACT

Aims: This study aims at comparative identification of antibiotic resistance patterns in bacteria isolated from samples collected from rural environment (LS) and urban environments (SS). Metagenomic profiling gave us insights into the microbial abundance of the two samples. This study focused on culture-based methods for complete identification of antibiotic resistant isolates and estimation of comparative antibiotic resistance among the two samples.

Study Design: Untreated medical waste and anthropogenic waste disposal can lead to the propagation of different antibiotic resistant strains in wastewater environments both in urban and rural set ups which provide an insight towards this study approach mentioned in the methodology segment.

Place and Duration of study: Sewer system of a medical facility located in Purulia, India was the collection site for liquid sludge. Solid sludge and associated wastewater were collected in vicinity of a large urban medical facility from central Kolkata, India.

Methodology: Physico-chemical properties were analyzed followed by microbiological and biochemical characterization. The antibiotic resistance patterns were determined by Kirby-Bauer disc diffusion assay. Potent multidrug resistant isolates were identified using 16srRNA gene amplification followed by Phylogenetic profiling, using CLC Genomics workbench.

Results: We observed maximum resistance in an *E. coli* isolate which was resistant up to 22 antibiotics. Combined data for resistance from urban and rural samples were found to exhibit 83.9% resistance to beta lactams, 85.7% to macrolides, 44.2% to fluoroquinolones, 50% to glycopeptides and cephalosporins, 35.7 % to carbapenems and sulfonamides, 28.5 % to tetracycline, and 23.8 % to aminoglycosides.

Conclusion: The high prevalence of antibiotic-resistant bacteria harbouring diverse resistance traits across samples indicated towards probable horizontal gene transfer across environmental niches. This study can prove to be useful to understand and map the patterns of resistance and stringently apply the counter measures related to public health practices.

Keywords: Antibiotic resistance, ARB, Environmental resistome, Antibiotic pollution, Wastewater, Sludge.

1. INTRODUCTION

The wonder molecules which were once used as lifesaving drugs have gradually been rendered ineffective by the evolution of microorganisms which have devised mechanisms of

resistance against them. Extensive use of antibiotics in healthcare and veterinary sectors along with their continuous unmonitored discharge in natural environments have successfully built up a reservoir of antibiotic resistant bacteria (ARB) which co evolve by acquisition of antibiotic resistance genes (ARG). This has led the world health organization to identify pathogenic strains of microorganisms which are potential superbugs exhibiting patterns of multidrug resistance thus directly diminishing the therapeutic potential of the antibiotics [1].

In India there is tendency of overuse of broad-spectrum penicillins owing to their availability and low cost. As a result, recent reports suggests that it is the leading country in per person antibiotic consumption which automatically results in the buildup of drug resistant pathogens [2] leading to the coinage of the phrase 'Antimicrobial resistance (AMR) capital of the world'[3].

There are several underlying factors such as inappropriate disposal of antibiotics, misuse etc. which cause intake and transfer of antibiotic resistant gene/genes in resistant bacteria [4]. The load of antibiotic resistance genes in natural environments contributes towards efficient transfer of these gene modules or collection of antibiotic resistant genes to different bacterial strains using mechanisms such as horizontal gene transfer, recombination or mutations under the selection pressure of different pollutants such as pesticides and heavy metals [5]. Some bacteria have been reported to utilize the antibiotic residues present as potential carbon source [6]. In ecological niches, the resistant genes are pooled together and as a result of this, there is an uptake of resistant genes among the bacterial neighbours horizontally. The lack of record of contamination is evident from the current reports of bacterial resistance towards third generation beta lactams and fourth generation cephalosporins [7]. There have been few reports on a lesser-known phenomenon, "environmental resistance loop" which refers to the transfer of resistant bacteria and antibiotic residues from wastewater treatment plants to the riverine systems and agricultural lands, finally reaching back to human beings and animals [8]. There is a growing concern that this phenomenon could render current and future antibiotics ineffective. According to current literatures, the mobile resistant genetic elements such as insertion sequences, transposons, integrons, and plasmids from clinical samples have been detected in hospital associated wastewaters, within a very minimal time interval following their report in hospitals [9, 10]. The unique characteristics of resistome are quite evident and thus there is a dire need for an integrated approach which includes effective wastewater treatment and continuous monitoring of the resistome. The main anthropogenic sources of dissemination of ARBs are effluents from wastewater and hospital discharge where horizontal gene transfer is very dominant [11] and as a result, the "difficult to treat" infections are increasing globally [12]. The causative agents and their representative antibiotic resistance profiles are present in low percentages in wastewaters, as compared to that in clinical settings. However, this presents a serious public health issue.

We still are in the nascent stage of our understanding regarding the types and prevalence of antibiotic resistance in the environment. Though, the standardization and organization of antibiotic resistance data of clinical origin has been curated, information regarding resistance of environmental bacteria is still very fragmented [13]. In India, according to the Resistance map resource [14] microbes resistant to aminoglycosides, carbapenems and cephalosporins pose the highest threat and have shown a steady pattern of increase from 2008 to 2020. The current pandemic has also opened a Pandora's box in terms of antibiotic usage, recent data from five different countries, suggests that there are associated bacterial infections in 6·9% of COVID-19 diagnoses of which 3·5% have been diagnosed during initial hospitalization while 14·3% post recovery. This trend is higher for patients who have received intensive critical care thus nosocomial infections cannot be ruled out [15]. This multicenter US based study also reported that almost 72% of the COVID patients were treated with antibiotics, even when there was no absolute clinical necessity [15]. The status of use of antibiotics in less developed countries are so scratchy that it is difficult to understand the actual burden that is being added to the environment as each day passes. Researchers believe that antimicrobial resistance might become even worse after COVID-19 due to their excessive use of antibiotics in humans, misuse in agriculture, and the unavailability of new formulations in the pipeline.

The aim of this study was to identify the diverse antibiotic resistant bacteria profiles in environmental wastewaters with respect to multiple antibiotics from diverse classifications. In a previous study the bacterial abundances of these two sites were reported using comparative metagenomics [16]. The metagenomic profiling gave us insights into the microbial abundance of the two samples. This study focused on culture-based methods for complete identification of antibiotic resistant isolates and estimation of comparative antibiotic resistance among the two samples.

2. MATERIAL AND METHODS

Wastewater environments were initially identified for their proximity to large medical facilities and then sample was collected as described in the previous report [16]. The microbiological culturing was initiated within 10 hours of collection. Stock samples were stored at 4°C for further processing.

2.1 Study area

The first area of this study is Purulia. Its latitudinal and longitudinal extents are from 22°42´35´´to 23°42´00´´North and from 85°49´25´´to 86°54´37´´East. The region is climatically characterised as a dry and arid zone with parts of the Chotanagpur plateau region contributing towards its warm and humid conditions. The sample collection site was wastewater effluent collected in vicinity of a medical facility located in Purulia. The climatic conditions were recorded; Temperature: 42°C, Relative humidity: 68%, Time of collection: 12 pm.

The second area of this study, Kolkata, is located in the eastern part of India. The Calcutta Municipal Corporation has an area of 185 sq km in total. A large part of the city historically was marshy wetlands, remnants of which can still be found especially towards the eastern parts of the city [17]. The sample collection site was solid sludge and associated wastewater collected in vicinity of a large urban medical facility located in central Kolkata. The climatic conditions were recorded; Temperature: 34°C, Relative humidity: 95%, Time of collection: 11:45 am.

2.2 Evaluation of physicochemical parameters of the sampling stations

The physico-chemical parameters of the effluents were analyzed as per standardized EPA protocols [18]. The parameters analyzed were Chemical oxygen demand (COD), Total organic carbon (TOC), Nitrate, Ammonia, total Kjeldahl nitrogen, total Phosphorus and heavy metals, viz. Chromium (Cr), Mercury (Hg), Lead (Pb), Cadmium (Cd), Arsenic (Ar).

2.3 Microbiological characterization

The effluent samples were serially diluted and plated onto nutrient agar plates (pH 6.6) and were incubated overnight at 37°C. Isolated colonies in higher dilution were observed on each type of plates. These colonies were then sub-cultured in nutrient agar plates for pure colony isolation and subsequently Gram stained. The colonies were observed under 100X Magnification using Bright Field Light Microscope for morphological identification. The Gram positive and Gram-negative bacterial isolates were then presumptively identified through a series of cultural and biochemical tests according to the criteria described in Bergey's Manual of Determinative Bacteriology [19].

2.4 Biochemical characterization

The isolates were subjected to routine biochemical tests: Catalase, Oxidase, Nitrate reduction, Indole, Methyl red, Voges-Proskauer, Citrate utilization, Urease, Starch hydrolysis, Carbohydrate fermentation [20].

2.5 Antibiotic resistance profiling

To determine the susceptibility of the isolates towards different antibiotics Kirby-Bauer disc diffusion assay was done. The antibiotic against which the susceptibility of the isolates was tested was selected on the basis of recommendations given in CLSI 2019, antibiotics belonging to diverse classifications and generations were used to check the sensitivity of the isolates. (Supplementary Table B). Disc diffusion assays were done using antibiotic discs

from HiMedia Laboratories (Mumbai, India). Sensitivity of the isolates against antibiotics (intermediate or susceptible) was deduced on the basis of the information given in the manufacturer's manual. Results were interpreted on the basis of CLSI guidelines [21]. We classified an isolate to be multi drug resistant (MDR) if it exhibited resistance to antibiotics belonging to three or more classes.

2.6 Molecular identification

Genomic DNA was isolated from different bacterial isolates using standard protocols and 16SrDNA fragment were amplified using universal 16S primers 27f (5′AGAGTTTGATCCTGGCTCAG3′) and 1492r (5′TACGGTTACCTTGTTACGACTT3′) [20], and sequenced. Raw sequences were assembled using the Cap3 Contig Assembly –in silico program. All the sequences were used to identify the bacteria with the help of the BLASTn program and were submitted to GenBank [22].

2.7 Phylogenetic analysis

For constructing the phylogenetic tree, initially two programs were used to generate the multiple sequence alignment for a rapid validation of sequence scores -they were Clustal omega [23] and Muscle [24]. Based on the consensus multiple sequence alignment, phylogenetic tree was determined with 100 bootstrap replicates [25] using the phylogenetic tree module of the CLC Genomics workbench. Here both neighbour joining-based methods [26] and maximum likelihood [27] based methods were tested to evaluate the tree generated and the best consensus tree was chosen for analysis and interpretation. The tree file was visualized in FigTree to distinguish the different cl

3. RESULTS AND DISCUSSION

3.1 Physico-chemical characterization

The physico-chemical parameters of the effluents were identified with the purpose of observing driving factors behind widespread antibiotic resistance and horizontal gene transfer. Heavy metals such as Cd, Pb, Hg were found to be higher than the permissible limits in both effluents (Fig. 1a). Heavy metals have been reported to be co-selecting agents, which promote resistance by co-resistance and cross-resistance mechanisms [28]. Total Phosphorus was higher than the permissible limits. COD was also found to be higher than the permissible limits (Fig. 1b).

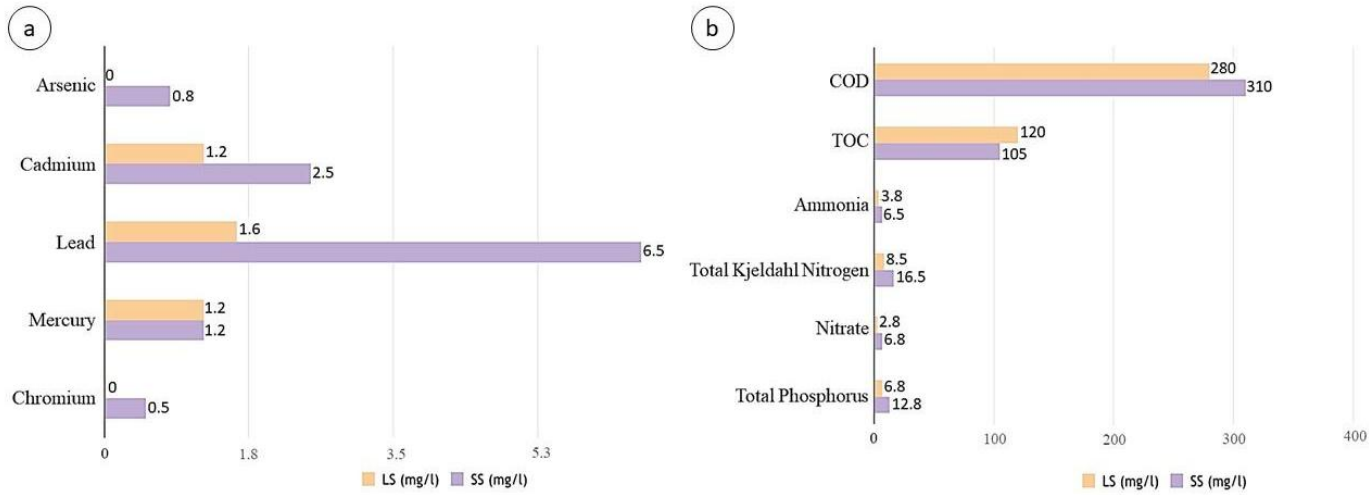

Fig. 1. Heavy metal concentrations in samples (Graphical representation of the estimation of heavy metal estimation in LS and SS sample sets with parameters on y-axis and concentrations (mg/l) on x-axis) (a) and Physico-chemical parameters of samples (Graphical representation of concentration of various analytes for LS and SS sample sets with parameters on y-axis and concentrations (mg/l) on x-axis) (b)

3.2 Microbiological characterization

The total number of isolated bacteria for LS and SS datasets was 19 and 23 respectively. The study was focused on multidrug resistant strains due to it was narrowed down to 14 isolates with 10 isolates from LS dataset and 4 isolates from SS dataset. Among the selected isolated bacteria, 8 were Gram negative rods (LS 2, LS 4, LS 6, LS 9, LS 17, LS 21, SS 7 and SS 19) and 6 were Gram-positive rod-shaped bacteria (LS 12, LS 14, LS 21, LS 23, LS 25 and SS 20).

3.3 Biochemical characterization

The isolated bacteria were subjected to various biochemical tests and the results were tabulated (Supplementary Table A and C).

3.4 Antibiotic resistance patterns

The antibiotic resistance was determined in terms of zone of inhibition (cm) data and it was found that isolates obtained from Purulia (rural area) was found to be much higher than that in Kolkata (urban area). *E. coli* isolate (LS 2) was resistant against 22 antibiotics out of 24 antibiotics tested (Supplementary Table B) which is in conformation with the public data released by ICMR. The percentage of resistance was higher in *E. coli* isolates compared to other isolates. Some *E. coli* isolates (LS 2, LS 9, LS 17) showed variable pattern of susceptibility which may be attributed to its higher relative abundance in fecal contaminated environments or increased competence towards receiving resistant gene containing foreign plasmids [29]. A study from diarrhoea affected children in Mexico revealed 73% isolates of *E. coli* 73% resistant to ampicillin [30]. In Pakistan, [31] identified a multi antibiotic resistant isolate of *E. coli* which was resistant to cefotaxime, ceftazidime, gentamycin, ciprofloxacin, imipenem. This study also exhibits similar patterns of resistance in *E. coli.* (Table 1) (Supplementary Table D)

207 **Table 1. Identified isolates with varying antibiotic resistance patterns**

Sample ID	Isolate	Antibiotics resistant pattern
LS 2	*Escherichia coli*	AMP, CIP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, VAN, ERY, MET, Cf, TET, DO, CTX, IPM, CXM, LE, COT, CX
LS 4	*Shigella flexneri*	AMP, CIP, NAL, AMC, Cd, E, MET, Cf, CXM
LS 6	*Klebsiella pneumoniae*	AMP, CIP, GEN, CTR, AK, AMC, OFX, NAL, AMC, Cd, E, MET,Cf, TET, DO, CTX, IPM, CXM, COT
LS 9	*Escherichia coli*	AMP, NOR, NAL, Cd, MET, Cf
LS 12	*Bacillus safensis*	AMP, Cd, ERY, MET, Cf, CXM
LS 14	*Bacillus australimaris*	AMP, NAL, AMC, Cd, VAN, ERY, MET, Cf, CX
LS 17	*Escherichia coli*	AMP, NAL, Cd, OFX, VAN, ERY, MET
LS 21	*Comamonas aquatica*	AMP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, ERY, MET, Cf, TET, DO, CTX, IPM, CXM
LS 23	*Lysinibacillus fusiformis*	AMP, CIP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, ERY, MET, Cf, TET, CTX, IPM, CXM, COT
LS 25	*Oceanobacillus caeni*	AMP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, VAN, ERY, MET, Cf, CTX, IPM, CXM, COT
SS 3	*Terribacillus halophilus*	AMP, VAN, ERY, Cf

SS 7	*Citrobacter freundii*	AMP, CIP, CTR, NAL, Cd, OFX, ERY, MET, Cf, TET
SS 19	*Comamonas aquatica*	AMP, NAL, Cd, VAN, MET
SS 20	*Bacillus pumilus*	AMP, CIP, CTR, NAL, AMC, Cd, MET, Cf, CXM

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209 *K. pneumoniae* is one of the commonest isolates in both hospital and community acquired
210 infections [32]. MDR and carbapenem resistant *K. pneumoniae* has become a major
211 therapeutic challenging scenario in several countries due to the lack of alternative existing
212 antibiotics. Manikandan and Amsath [33] observed high degree of resistance in *K.*
213 *pneumonia*e isolated from a urine sample. They reported resistance to ampicillin,
214 ceftazidime, cefotaxime, ciprofloxacin and gentamicin. In our study, *K. pneumonia* isolate
215 (LS 4) also exhibits resistance against beta lactams, fluoroquinolones, aminoglycoside,
216 macrolides, tetracycline, cephalosporins, carbapenems and sulphonamide.

217 Several reports from around the world specially, India, Canada, Israel, Argentina, Turkey,
218 Lebanon, Iran, China, Japan and South Korea, have reported *Shigella* spp. harbouring
219 different types of ESBL genes [34]. In our study we found that *Shigella flexneri* isolate (LS 2)
220 showed variable resistance towards beta lactams, fluoroquinolones, macrolides and
221 cephalosporins.

222 Several strains of the commonly occurring *Lysinibacillus fusiformis* have been isolated from
223 multiple environments including agriculture soil and factory wastewater [35]. However, there
224 are no reports corresponding to the emergence of multidrug resistance in it. *L. fusiformis* has
225 been reported to be sensitive to tetracycline previously [36]. In our study, *L. fusiformis* (LS
226 23) was found to be resistant against beta lactams, fluoroquinolones, macrolides,
227 tetracycline, cephalosporins and sulphonamide.

228 Bacteria of the *Bacillus pumilus* clade are ubiquitous in a wide variety of terrestrial and
229 marine environments, ranging from stratospheric air to deep-sea sediments and from soil to
230 living beings [37, 38, 39]. The following species belonging to *B. pumilus* clade: *B. pumilus*
231 *(*LS 16), *B. safensis* (LS 21) and *B. australimaris* (LS 14) were identified in this study. The
232 antibiotic resistance patterns of the sister clade isolates are quite similar in nature with
233 resistance shown against beta lactams, fluoroquinolones, macrolide and cephalosporins.
234 This presents an interesting insight into the evolutionary link of antibiotic resistance genes
235 across various species.

236 *Comamonas* species are occasional human pathogens found in contaminated environments
237 [40]. Their identification has been challenging with several laboratories ending up reporting
238 them as non fermentative gram-negative bacilli that could not be further identified [41]. We
239 have identified two *C. aquatica,* one each from both the samples (LS 17 and SS 20), which
240 show variable resistance against beta lactams, fluoroquinolones and glycopeptide class of
241 antibiotics. This is probably the first report of antibiotic resistance in *Comamonas* species
242 from a waste water environment.

243 *Oceanobacillus caeni* was first isolated in South Korea as a component of activated sludge
244 in a Bacillus-dominated wastewater treatment plant [42]. In our study the isolate (LS 22) was
245 obtained from rural wastewater and it showed variable resistance against beta lactams,
246 fluoroquinolones, macrolides, cephalosporins and sulphonamide. The pathology and
247 antibiotic resistance patterns of this species have not been conclusively studied before.

248 *Citrobacter freundii* is a frequent cause of nosocomial infections and a known cause of
249 diarrheal infections, has increasingly become multidrug resistant [43]. *C. freundii* isolate (LS
250 25) was identified and showed resistance against beta lactams, fluoroquinolones,
251 aminoglycoside, macrolides, cephalosporins and tetracycline.

Terribacillus halophilus was originally isolated from field soil in Japan [44]. The isolate (SS 3) showed variable antibiotic resistance patterns against beta lactams, glycopeptides, macrolide and cephalosporin. This is the first report of occurrence of antibiotic resistance in this species. (Figure 2a and b).

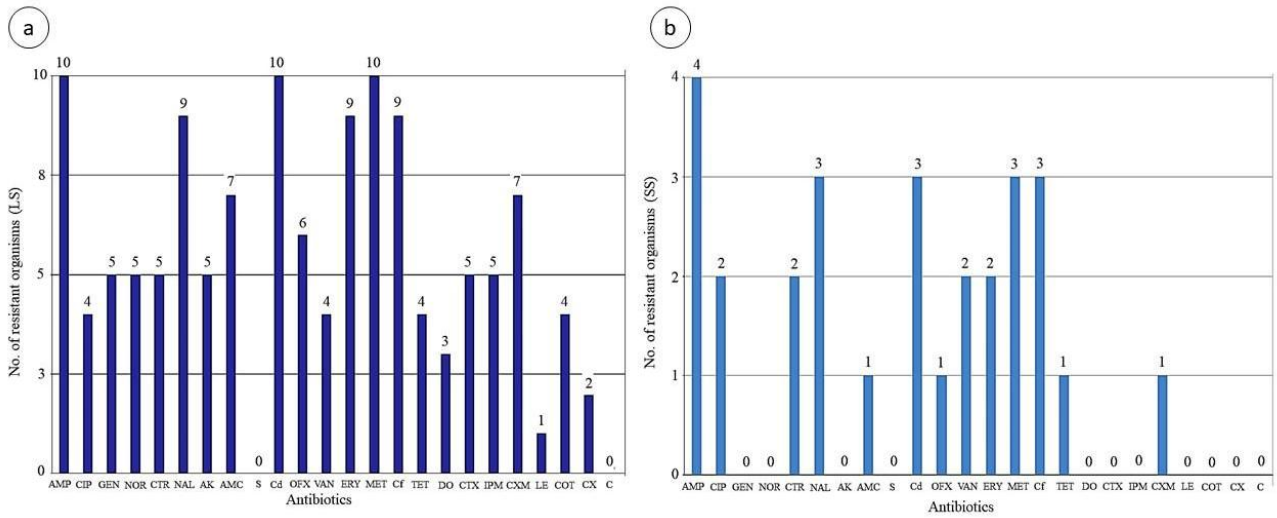

Fig. 2. Antibiotic resistant bacteria isolated from a rural setting in West Bengal, India (Graphical representation of the number of resistant isolates in LS sample set against various antibiotics; the number of resistant isolates is represented on y-axis and the antibiotics on x-axis) (a) and Antibiotic resistant bacteria isolated from an urban setting in West Bengal, India (Graphical representation of the number of resistant isolates in SS sample set against various antibiotics; the number of resistant isolates is represented on y-axis and the antibiotics on x-axis) (b)

This study identifies 83.9% of the isolates to exhibit resistance to beta lactams with 100% resistance to Ampicillin. [45] reported higher probability of occurrence of ESBL-producing *K. pneumonia* and *E. coli* isolates in hospital effluent, Waste Water Treatment Plants (WWTP) and river samples, respectively whereas, hospital effluent, sanitary effluent, outflow sewage and surface water samples were richer in quinolone resistant isolates. In this study, high macrolide resistance among the isolates (85.7%) which clearly points to the diverse antibiotic resistance potential of the effluents. The resistance to fluoroquinolones was 44.2 %, 50% for glycopeptides and cephalosporins, 35.7 % for carbapenems and sulfonamides, 28.5 % for tetracycline, 23.8% for aminoglycosides. All the isolates were however, found to be susceptible to chloramphenicol and streptomycin which may be due to the fact that these antibiotics are prescribed very sparsely by healthcare professionals and medical practitioners in the vicinity of the collection spots.

Phylogenetic profiling revealed the presence of 4 distinct phylogenetic clusters (Table 2) (Figure 3). The largest cluster had 5 individual OTUs (LS 4, SS 7, LS 17, LS 9, SS 19). In this group, all the isolates showed resistance against AMP, NAL, Cd, VAN and MET antibiotics. It is interesting to note that this group contains isolates obtained from different geographical and sampling conditions which indicate the possibility of genetic exchange amongst allochthonous species [46]. The cluster containing isolates LS 6, LS 2, LS 21 show resistance against a wide range of antibiotics such as beta lactams, fluoroquinolones, aminoglycoside, macrolides, tetracycline, cephalosporins, carbapenems and sulphonamides, evolving probably through Darwinian forces [47]. The sister group with OTUs obtained from different sampling conditions such as LS 14, SS 20, LS 12 show resistance against beta lactams and cephalosporins both groups inhibiting bacterial cell wall synthesis. The cluster with OTUs LS 23, SS 3 and LS 25 contains halophilic organisms with resistance against some common antibiotics such as beta lactams and cephalosporins. LS 2 and LS 6 belong to the sister clades and exhibit similar antibiotic resistance patterns viz. resistance against beta lactams, fluroquinolones, aminoglycosides, macrolides, tetracycline, cephalosporins

and sulfonamide. LS 25 and SS 3 show resistance against beta lactams and glycopeptides again with the common mode of action, i.e. inhibition of cell wall synthesis. They are closely related members with respect to similarities in 16S rRNA gene sequences which justifies the observations of [48], that evolutionarily related bacteria have greater chance of being selected for in polluted environments either due to the presence of antibiotics or by the process of co selection of other pollutants (Figure 4).

Table 2. Description of isolates represented in the phylogenetic tree, along with their sample IDs and GenBank Accession numbers

Phylogenetic code	Sample ID	Accession number	Bacterial identity
Sample_1	LS 4	MW380613	*Shigella flexneri*
Sample_2	LS 6	MW380614	*Klebsiella pneumoniae*
Sample_3	LS 2	MW380615	*Escherichia coli*
Sample_4	LS 23	MW380616	*Lysinibacillus fusiformis*
Sample_5	LS 14	MW380617	*Bacillus australimaris*
Sample_6	SS 20	MW380618	*Bacillus pumilus*
Sample_7	LS 21	MW380619	*Comamonas aquatica*
Sample_8	LS 12	MW380620	*Bacillus safensis*
Sample_9	SS 3	MW380621	*Terribacillus halophilus*
Sample_10	SS 7	MW380622	*Citrobacter freundii*

Sample_11	LS 17	MW380623	*Escherichia coli*
Sample_12	LS 25	MW380624	*Oceanobacillus caeni*
Sample_13	LS 9	MW380625	*Escherichia coli*
Sample_14	SS 19	MW380626	*Comamonas aquatica*

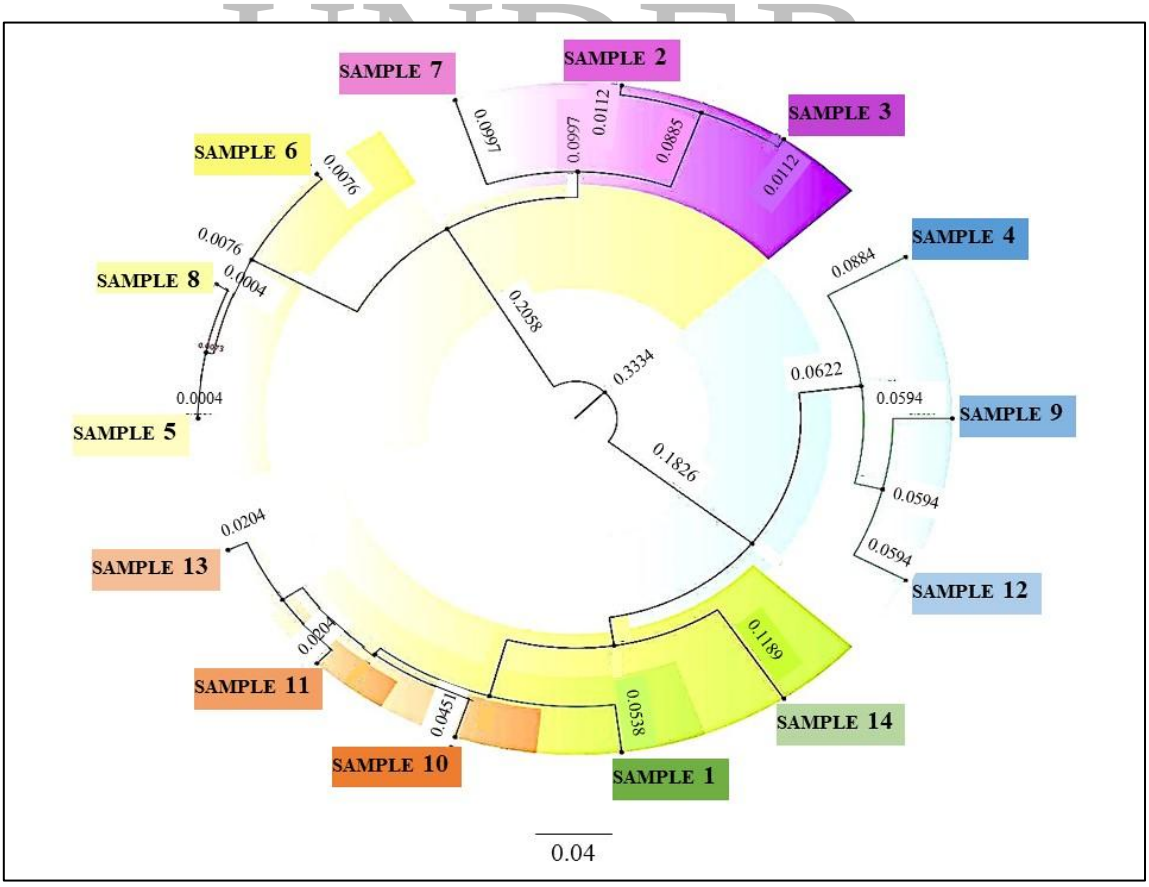

Fig. 3. Phylogenetic analysis. Four distinct clades were identified in the phylogenetic tree (Clade 1 -LS 4, SS 7, LS 17, LS 9, SS 19; Clade 2- LS 6, LS 2, LS 21; Clade 3- LS 14, SS 20, LS 12; Clade 4- LS 23, SS 3 and LS 25

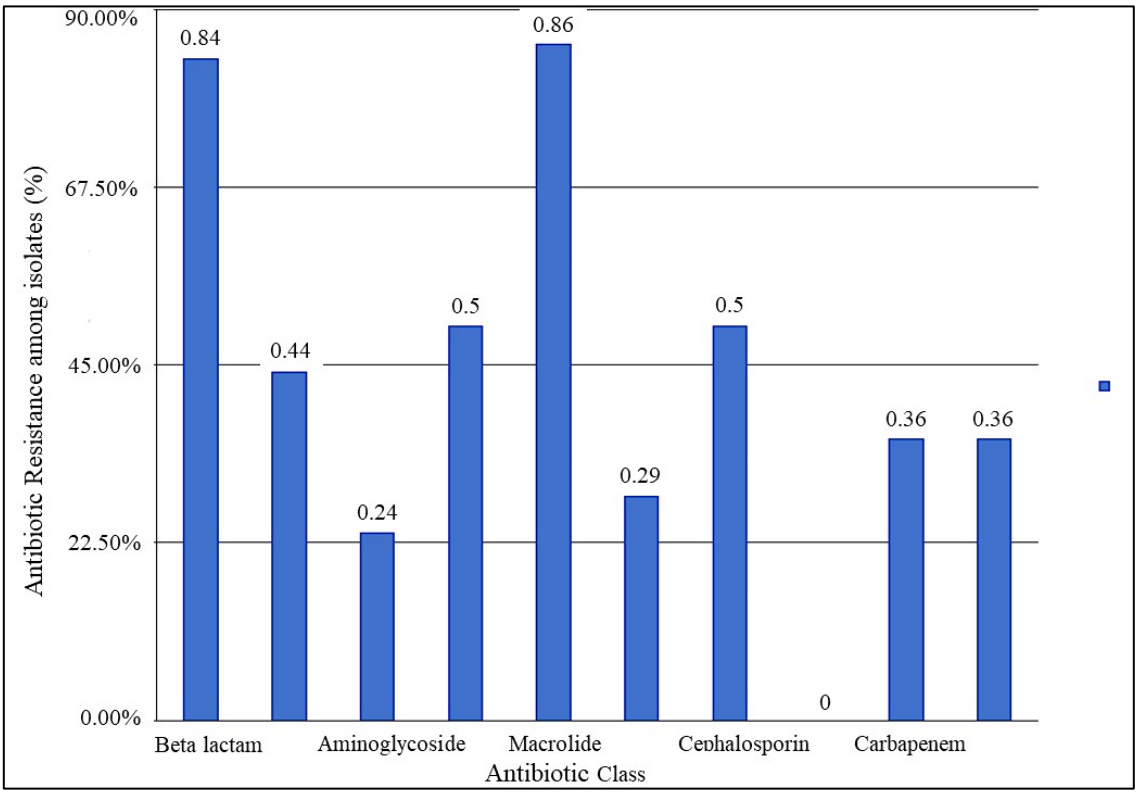

Fig. 4. Antibiotic resistance patterns of bacteria isolated from wastewaters collected from rural and urban areas of West Bengal, India. (Graphical representation of % antibiotic resistance on y-axis plotted against various classes of antibiotics on x-axis)

This study brings to light three important insights towards the ever-increasing burden of antimicrobial resistance in the environment, specially in wastewater. The first is the identification of multidrug resistant *E.coli* which has been included as one of the most important AMR indicators along with methicillin-resistant *Staphylococcus aureus* (MRSA) from 2019 onwards. WHO reports (https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance) suggests that 25 countries, territories and areas have provided data to the Global Antimicrobial Resistance and Use Surveillance System (GLASS) on blood-stream infections due to MRSA and 49 countries provided data on bloodstream infections due to *E.coli where* the median rate observed for methicillin-resistant S. aureus was 12.11% with an inter quartile range (IQR) of 6.4–26.4 and that for *E. coli* resistant to third generation cephalosporins was 36.0% (IQR -15.2–63.0). Thus the multidrug resistant isolate obtained from this study can also provide important insights towards the resistant gene profiles if studied further. The second important isolate exhibiting resistance is *Lysinibacillus fusiformis* which have been reported in environmental samples but with very little resistant properties. Nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) have been isolated from *Lysinibacillus fusiformis* both of which regulate the synthesis of antimicrobial compounds in the organism [49]. Thus the development of multidrug resistance in the isolate obtained in this study, probably indicates towards a mechanism in which the bacteria is able to survive competition in a particular wastewater niche as well as fortify its drug resistance mechanisms. The third interesting isolate is *Commamonas aquatica,* found in both urban and rural samples and resistant to beta lactams which has not been reported earlier. *However,* recent reports suggest the presence of antibiotic resistant *Commamonas testosteroni* in hospital set ups and in acute appendicitis [50] . It seems that the bacteria is increasing its resistance repertoire which is alarming in terms of the potential threat which it might pose in the coming years.

4. CONCLUSION

The results obtained in our analyses, leads us to believe that untreated medical waste and anthropogenic waste disposal can lead to the propagation of different antibiotic resistant strains in wastewater environments both in urban and rural set ups. It is quite evident that the isolates from rural area showed high rates of resistance as compared to that of urban area. Excreted antibiotics can end up in wastewater treatment plants which are capable of degrading the compound only partially. At the same time, such facilities probably serve as hotspots of horizontal gene transfer between bacterial species and even a few such strains if released in the environment can contribute negatively [51]. This reflects on the lack of awareness towards proper antibiotic usage in rural areas as compared to that in urban areas. This reflects on the need to practice efficient waste disposal and wastewater treatment policies. In natural settings, bacteria harboring antibiotic resistance genes are part of complex communities where they interact with other species [52].

This study should enable us to understand the route of spread of antibiotic resistant isolates in urban and rural wastewaters thus necessitating public awareness regarding measures of hygiene. Further, it should also enable us to formulate a monitoring program for evaluating wastewater health using a combination of culture independent and dependent molecular techniques to limit the spread of these emerging contaminants under tropical conditions.

CONSENT: Not Applicable

ETHICAL APPROVAL: Not Applicable

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SUPPLEMENTARY INFORMATION:

Biochemical characterization

Supplementary Table A. Biochemical tests for phenotypic characterization

Name of test	Purpose
Catalase test	To identify organisms those produce the enzyme, catalase which detoxifies hydrogen peroxide by breaking it down into water and oxygen gas.
Oxidase test	To identify microorganisms containing the enzyme cytochrome oxidase.
Nitrate reduction test	To determine the production of an enzyme called nitrate reductase, which results in the reduction of nitrate (NO_3).
Indole test	To screen for the ability of an organism to degrade the amino acid tryptophan to produce indole.
Methyl red test	To determine whether the microbe perform mixed acids fermentation when supplied glucose.
Voges-Proskauer test	To detect acetoin in a bacterial broth culture.
Citrate utilization test	To test an organism's ability to utilize citrate as a source of energy by the use of citrate agar.
Urease test	To identify bacteria capable of hydrolyzing urea using the enzyme urease.
Starch hydrolysis test	To identify bacteria that can hydrolyze starch (amylose and amylopectin) using the enzymes a-amylase and oligo-1,6-glucosidase.
Carbohydrate fermentation test	To determine the utilization of a certain carbohydrate, which results in acid or gas production after carbohydrate fermentation.

514 **Antibiotic resistance profiling**

515 **Supplementary Table B. Antibiotic classification and mechanisms**

Class	Mechanism	Antibiotic	Abb.	Conc. (µg)
Beta lactam	Inhibition of cell wall synthesis.	Ampicillin	AMP	10
		Methicillin	MET	30
		Amoxyclav	AMC	30
		Cephalothin	Cf	30
Fluoroquinolone	Interruption of DNA breakage-reunion step by binding DNA-gyrase or topoisomerase II and topoisomerase IV.	Ciprofloxacin	CIP	10
		Norfloxacin	NOR	10
		Ofloxacin	OFX	5
		Nalidixic acid	NAL	30
		Levofloxacin	LE	5
Aminoglycoside	Inhibition of protein synthesis by binding to 30S ribosomal subunit.	Amikacin	AK	30
		Streptomycin	S	10
		Gentamicin	GEN	10
Glycopeptide	Inhibition of cell wall synthesis.	Vancomycin	VAN	30

Macrolide	Inhibition of bacterial protein synthesis.	Erythromycin	ERY	15
		Clindamycin	Cd	2
Tetracycline	Inhibition of bacterial protein synthesis.	Tetracycline	TET	30
		Doxycycline	DO	30
Cephalosporin	Inhibition of cell wall synthesis.	Ceftriaxone	CTR	30
		Cefoxitin	CX	30
		Cefotaxime	CTX	30
		Cefouroxime	CXM	30
Chloramphenicol	Inhibition of bacterial protein synthesis.	Chloramphenicol	C	30
Carbapenem	Inhibition of cell wall synthesis.	Imipenem	I	10
Sulfonamides	Inhibition of bacterial folic acid synthesis	Co-trimoxazole	COT	25

Biochemical characterization

Supplementary Table C. Biochemical test results for bacteria isolated from wastewater samples (C: Catalase; O: Oxidase; NR: Nitrate reductase; I: Indole;MR: Methyl red; VP: Voges-Proskauer; CU: Citrate utilization; U: Urease;

SU: Starch utilization; G: Glucose; l: Lactose; S: Sucrose; -: Negative; +: Positive)

Sample ID	C	O	NR	I	MR	VP	CU	U	SU	Carbohydrate fermentation		
										G	L	S
LS 2	+	-	+	+	+	-	-	-	+	+	+	+
LS 4	+	-	+	-	+	-	-	-	-	+	-	-
LS 6	+	-	+	-	-	+	+	+	-	+	+	+
LS 9	+	-	+	+	+	-	-	-	+	+	+	+
LS 12	+	+	-	-	-	+	-	-	+	+	-	+
LS 14	+	+	-	-	+	+	+	+	-	+	-	+
LS 17	+	-	+	+	+	-	-	-	+	+	+	+
LS 21	+	+	-	-	+	+	-	-	-	-	-	-
LS 23	+	-	-	-	-	-	-		+	-	-	-
LS 25	-	+	-	-	+	-	-	+	-	+	-	+
SS 3	+	+	+	-	-	+	-	+	+	+	-	+
SS 7	+	-	+	-	+	-	+	+	-	+	+	+

SS 19	+	+	-	-	+	+	-	-	-	-	-	-
SS 20	+	+	-	-	-	-	-	-	+	-	-	-

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525 **Supplementary Table D. Concentrations of antibiotics used for testing antibiotic resistance patterns**

Antibiotic	Abbreviation	Concentration (µg/µl)
Ampicillin	AMP	10
Methicillin	MET	30
Amoxyclav	AMC	30
Cephalothin	Cf	30
Ciprofloxacin	CIP	10
Norfloxacin	NOR	10
Ofloxacin	OFX	5
Nalidixic acid	NAL	30
Levofloxacin	LE	5
Amikacin	AK	30
Streptomycin	S	10
Gentamicin	GEN	10
Vancomycin	VAN	30
Erythromycin	ERY	15
Clindamycin	Cd	2
Tetracycline	TET	30

Doxycycline	DO	30
Ceftriaxone	CTR	30
Cefoxitin	CX	30
Cefotaxime	CTX	30
Cefouroxime	CXM	30
Chloramphenicol	C	30
Imipenem	I	10
Co-trimoxazole	COT	25