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Antibiotic resistance in a predominantly occurring Gram-negative bacterial community from treated sewage to assess the need for going beyond coliform standards

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ABSTRACT

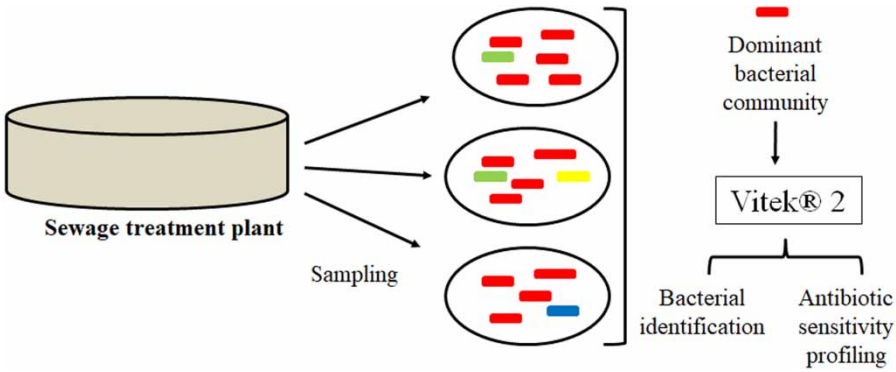
Antibiotic resistance surveillance is an objective of global action plan on antimicrobial resistance endorsed by the World Health Organization. The current study reports the identification of frequently occurring Gram-negative bacterial community (GNBC) previously isolated from municipal treated wastewater and their antibiotic resistance profiles. Further, the genes responsible for extended-spectrum beta lactamases (ESBL) activity were identified in ESBL-positive organisms. The isolates were characterized using biochemical assays and identification was confirmed by VITEK®2 automated system. Antibiotic susceptibility testing against seven different classes of antibiotics was also performed on the same system using AST-N280 cards. The most dominant isolates identified were *Acinetobacter baumannii*, *Morganella morganii*, *Kluyvera intermedia*, *Stenotrophomonas maltophilia*, *Escherichia coli*, *Aeromonas hydrophila/caviae*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Citrobacter freundii*. The isolates were observed to be significantly resistant against the antibiotics amoxicillin-clavulanic acid, cefuroxime, cefuroxime axetil and colistin. Two of the isolates, *E. cloacae* sp. dissolvens and *S. maltophilia*, were found to be positive for ESBL activity encoded by *blaCTX-M* gene. The possible intrusion of hospital wastewater in domestic sewage is also discussed. This study may help assess the risk of wastewater reuse by detecting dominant bacteria as a step towards the development of new microbiological standards.

Key words: ESBL, Gram-negative bacterial community, multiple drug resistance, treated municipal sewage, VITEK®2

HIGHLIGHTS

- *Stenotrophomonas maltophilia* found to be most resistant and *E. coli* least resistant among the isolates.
- Five isolates found to be resistant to the last resort antibiotic Colistin.
- Molecular determinant of two extended spectrum beta lactamase producing isolates found to be *blaCTX-M* gene.
- This report might aid in devising a suitable strategy for reducing risk to human health upon treated wastewater reuse.

GRAPHICAL ABSTRACT



ABBREVIATIONS

ARB	Antibiotic-resistant bacteria
ARG	Antibiotic resistance gene
ESBL	Extended-spectrum β-lactamases
GNBC	Gram-negative bacterial community
MDR	Multiple drug resistance
MLD	Millions litres per day
STP	Sewage treatment plant
SMX-TMP	Sulfamethoxazole-trimethoprim

1. INTRODUCTION

Over the last few years, the emergence and dissemination of antibiotic resistance among bacteria has become a serious public health concern throughout the world (Rabbani *et al.* 2017). In developing countries, sewage treatment plants are designed primarily for the removal of organic pollutants and the microbiological contaminants are not considered of much importance in such treatment processes (Bhatt *et al.* 2020). Traditionally, the emergence of antibiotic resistance has been perceived as a clinical problem, but currently, non-clinical environments have revealed important factors in the spread of antibiotic resistance genes (ARGs) (Berglund 2015). Sewage treatment plants (STPs) have now emerged as a major source of antibiotic release into the environment. They also serve as a potential hotspot for horizontal gene transfer and selection of antibiotic-resistant bacteria (ARB). It has been estimated that almost 99% of total bacterial community in treated wastewater could have the potential of any type of antibiotic resistance acquisition (Rizzo *et al.* 2013).

In the absence of strict implementation of wastewater discharge norms, inadequately treated wastewater from various sources, including industries and hospitals, may enter municipal wastewater treatment plants (Chagas *et al.* 2011). A recent study has indicated that treated/partially treated wastewater from hospitals is being discharged in the centralized municipal wastewater treatment plants in Jaipur (Bhatt *et al.* 2020). Many studies have reported that pharmaceutically active substances, antibiotics, nosocomial pathogens, ARBs and plasmid-mediated ARGs are not effectively eliminated during sewage treatment (Chagas *et al.* 2011). In another study, the surveillance of the applications of disinfectants and other antimicrobials for prevention of co-resistance development in multiple drug-resistant (MDR) nosocomial pathogens has been recommended (Kumar *et al.* 2020). Although Gram-negative pathogens as well as indicator bacteria such as *Escherichia coli* and *Klebsiella* have been reported to be the most dominant bacteria, MDR has been observed in more than 50% of these bacterial populations (Rabbani *et al.* 2017). A published report on antimicrobial resistance in India revealed that maximum studies have been reported from humans and less than 5% have been reported from the environment category. The report further recommended a wider reporting on antibiotic resistance in bacteria from different environmental settings/origins to understand the role of environment for tackling the AMR challenge (Gandra *et al.* 2017).

Generally, indicator organisms such as faecal and total coliforms are routinely quantified for the possible presence of pathogens in water (WHO 2017). It has been reported that the microbiological quality of final effluent is strongly influenced by different treatment facilities rather than being dependent on influent characteristics. On the other hand, the lack of correlation between indicator and pathogenic bacteria raises a question about the existing standards (Bhatt *et al.* 2020). Therefore, routine testing of indicator organisms is not sufficient to confirm the presence of MDR bacteria. This assumes even greater importance in light of recent findings that indicate high MDR being present along with high antimicrobial as well as disinfectant resistance in nosocomial opportunistic pathogens (Kumar *et al.* 2020). Further, if the treated sewage is used for agriculture, which is commonly practised around the world, relying on the faecal coliform standards may magnify the biological risk to the health of humans.

The composition of microbial community in wastewater is too diverse and complex to understand the infection risks at the receiving end. Therefore, development of a presumptive method to quickly assess the dominant bacterial presence and possible risks (antibiotic resistance and others) based on culture-dependent techniques may be beneficial for low-income countries. Although the less abundant members of the bacterial community can also be important as carriers of ARGs (that might become dominant under appropriate selection pressures), an analysis of dominant members present at a

particular time is likely to provide the clue about prevailing selection pressures and the selected organisms.This type of study can serve as a quick method to understand the presumptive risks associated with the treated effluent.

In the current study, the dominant Gram-negative bacterial community (GNBC) was selected. Infections caused by GNBC are of particular concern as these communities are accountable for a broad range of hospital-acquired infections including urinary tract infections, pneumonia and bacteraemia (Talbot *et al.* 2006). At the same time, the Gram-negative infections have been reported to be linked with a substantially greater rate of mortality (Makhoul *et al.* 2005). Therefore, the spread of antibiotic resistance in GNBC is a major concern and challenge. These infections are generally managed in hospitals by administration of beta-lactam antibiotics. Resistance to beta-lactam antibiotics is frequently observed due to production of beta-lactamases. The extended-spectrum beta-lactamases (ESBLs) impart resistance to penicillin, first, second and third-generation cephalosporins and aztreonam. The most prevalent ESBL genes are *bla*-SHV, *bla*-TEM and *bla*-CTX-M (Adelowo *et al.* 2018).

In the current study, the predominantly occurring Gram-negative bacterial population from wastewater has been characterized and its antibiotic resistance profiling as an emerging risk for public health and environment safety has also been performed. The strains exhibiting resistance against multiple beta-lactam antibiotics were further characterized using molecular tools to ascertain the gene(s) responsible for the resistance. The overall aim of this work was to assess the antibiotic resistance profile of the dominant Gram-negative population in order to evaluate whether the dominant population is likely to pose a risk to human health and contribute to the spread of antibiotic resistance. If so, which groups of bacteria are responsible for the associated risk? The ultimate goal was to assess whether the present practice of assessing the coliform counts in treated water will be adequate to ensure public health and safety or if there is a requirement of more detailed microbial assessments for testing the efficacy of sewage treatment plants.

2. MATERIALS AND METHODS

2.1. Site of study

The study was performed on samples from a sewage treatment plant (moving bed bioreactor) located at Jawahar circle, Jaipur, India (Figure 1). This plant has a capacity of 1 million litres per day and it receives municipal sewage. The treated wastewater is used for irrigating the adjoining garden at Jawahar circle, Jaipur, itself.

2.2. Bacterial isolates

Three samples were collected in November 2018 and were analysed for the presence of culturable Gram-negative bacteria on MacConkey's agar plates (Shekhawat *et al.* 2020a). The most common and frequent culturable bacterial colonies (from three samples and their replicate) were selected as dominant bacteria. These dominant Gram-negative bacterial isolates were serially diluted and plated with 100 μL of each dilution on MacConkey agar incubated (37 °C, 24 to 48 hours) for pure bacterial culture preparations, species identification and their antibiotic resistance profiling.

2.3. Biochemical analysis

Biochemical tests such as indole, Methyl red, Voges-Proskauer, citrate, triple-sugar-iron agar, and catalase and oxidase were carried out following the standard protocols (Bailey & Scott 1962). Biochemical tests were performed as routine for the initial screening of the isolates to get an idea about their taxonomic classification and to discriminate between species showing similar colony morphologies to facilitate selection of strains for VITEK®2 analysis.

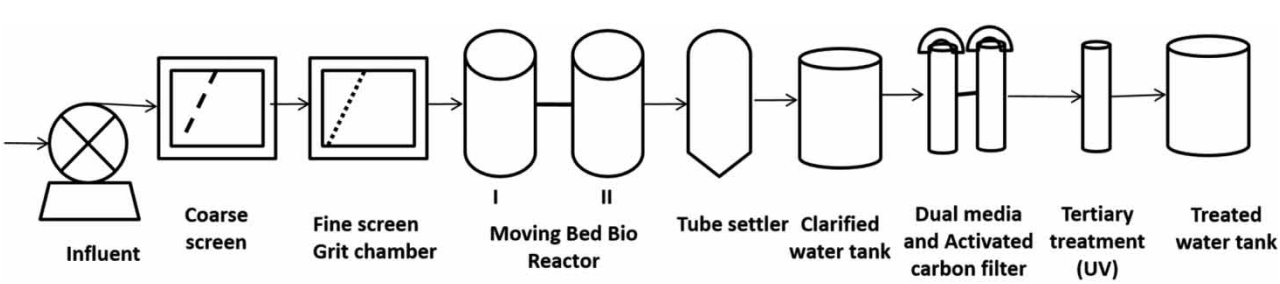


Figure 1 | Schematic diagram of sewage treatment plant located at Jawahar circle, Jaipur.

2.4. Identification of the isolates and their antibiotic susceptibility using VITEK®2 system

The identification and antibiotic susceptibility analysis of the isolates was carried out using the VITEK®2 compact automated microbial system (Manyahi *et al.* 2014). Identification of bacteria was performed using ID-GN Vitek® cards (bioMérieux). Vitek® cards were selected on the basis of Gram staining. This system is based on API technology that comprises 64 tests and is automated. Antibiotic susceptibility testing was performed using AST-N280 cards. The isolates were classified as resistant or sensitive on the basis of their minimum inhibitory concentrations following standard Clinical and Laboratory Standards Institute guidelines (CLSI 2018). This is the most commonly used method in clinical settings, and is highly reliable, cost effective, easy to use and least labour-intensive system (Manyahi *et al.* 2014).

2.5. Screening for extended-spectrum β-lactamase

ESBL production was confirmed by phenotypic confirmatory disc diffusion test. Cefotaxime (CTX) (30 μg) and cefotaxime/clavulanic acid (CEC) 10/30 μg discs were placed on Muller-Hinton agar swabbed with the test organism. Increase in zone diameter of ≥5 mm in the presence of CEC acid than cefotaxime alone was interpreted as positive for ESBL production (Figure 2). *E. coli* CDC 241, a known in-house ESBL producer, was used as positive control whereas *E. coli* 25922 was used as negative control. The test was done in accordance with the CLSI guidelines (CLSI 2018).

2.6. Identification of gene responsible for ESBL activity

Overnight grown cultures (Luria broth, 37 °C) of confirmed ESBL producers were subjected to plasmid DNA isolation using Plasmid DNA isolation kit (HiMedia, India) following the manufacturer's instructions. The quality of DNA was confirmed using spectrophotometer and agarose gel electrophoresis. PCR was carried out in 50 μL volumes containing 20 ng of template DNA, 0.5 mM dNTPs, 1.25 μM of forward and reverse primer specific for *blaCTX-M*, *blaSHV* and *blaTEM* genes (Paterson *et al.* 2003) and 3 μL Taq DNA polymerase in 1X PCR buffer. Amplification was performed in Master cycler (Eppendorf, Germany) with the following cycling parameters: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C, for 1 min; annealing at 61 °C for 1 min; extension at 72 °C for 1 min, followed by final extension at 72 °C for 5 min. PCR products were analysed on 2% agarose gel.

3. RESULTS AND DISCUSSION

3.1. Identification of the isolates

Eleven frequently occurring Gram-negative bacterial strains previously isolated from the treated wastewater were characterized using biochemical tests. The results of preliminary biochemical tests are depicted in Table 1. The bacteria showed distinct biochemical profile suggesting that they belong to different species of coliforms and non-coliforms. These isolates were further identified using VITEK®2 system. On the basis of results of VITEK®2, the isolates were identified as *Acinetobacter baumannii* (JPJC-S1), *Enterobacter cloacae* ssp. dissolvens (JPJC-S2), *Citrobacter freundii* (JPJC-S3), *Kluyvera intermedia* (JPJC-S4),

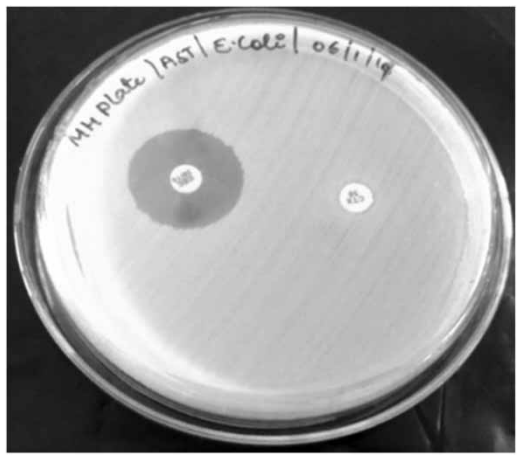


Figure 2 | Phenotypic confirmatory disc diffusion test showing an increase in zone size of >5 mm for cefotaxime/clavulanic acid.

Table 1 | Results of biochemical test for the 11 isolates

Test isolate	Indole	MR	VP	Citrate	Catalase	Oxidase	Urease	TSI Butt/Slant
JPJC-S1	−	+	−	+	+	−	−	K/K
JPJC-S2	−	−	−	−	+	−	+	AG/A
JPJC-S3	−	+	−	+	+	−	−	K/K, H_2S
JPJC-S4	+	+	−	−	−	−	−	AG/A
JPJC-S6	−	−	−	−	+	−	+	AG/A
JPJC-S7	+	−	−	−	+	−	−	AG/A
JPJC-S8	+	−	−	−	+	−	+	AG/A
JPJC-S9	−	+	−	−	−	−	+	AG/A
JPJC-S10	+	−	−	−	−	+	−	A/A
JPJC-S13	+	+	−	+	+	−	+	A/K
JPJC-C	−	−	−	−	+	+	+	K/K

MR, Methyl red; VP, Voges–Proskauer; TSI, Triple sugar iron. A/A, glucose and lactose and/or sucrose fermentation; K/K, no fermentation; AG/A, glucose and lactose and/or sucrose fermentation and gas produced; A/K, glucose fermentation only.

Morganella morganii ssp. morganii (JPJC-S6), *E. coli* (JPJC-S7 and S8), *Klebsiella pneumoniae* ssp. pneumoniae (JPJC-S9), *Aeromonas hydrophila/caviae* (JPJC-S10), *Enterobacter cloacae* ssp. cloacae (JPJC-S13) and *Stenotrophomonas maltophilia* (JPJC-C). Nine of the 11 isolates showed more than 93% confidence in identification. The isolate *A. hydrophila/caviae* showed 93% probability with low discrimination between species whereas the isolate *C. freundii* showed 87% probability (acceptable identification) in VITEK®2 results (Supplementary material, Table S1). In this study, three out of the 11 identified species, (*A. baumannii, M. morganii* and *K. intermedia*, have been listed in the WHO list of critical priority bacteria for which there is an urgent need for development of new antibiotics (WHO 2017). Therefore, it is suggested that the presence of critical priority bacteria among a few dominant bacteria may help to presume the relative risk. Moreover, this may help to devise a suitable treatment technology to inactivate the potential pathogens. In a previous work, the identification of three isolates by 16 S rRNA gene sequencing validated the specificity of VITEK®2 system as JPJC-S3, JPJC-S9 and JPJC-C were identified as *Citrobacter, Klebsiella* sp. and *S. maltophilia* by both the methods (Shekhawat *et al.* 2020a). Many of the bacterial isolates reported in this study have frequently been reported in hospital wastewater effluent and some of them are opportunistic pathogens (Zhang *et al.* 2013; Rabbani *et al.* 2017). However, no correlation was established for nosocomial origins. Recently, the removal efficiencies of different bacterial groups were shown to vary greatly. It was also noted that emerging pathogens are less likely to be removed as compared to indicator organisms; however, antibiotic resistance was not studied (Bhatt *et al.* 2020).

3.2. Antibiotic sensitivity profiling

The antibiotic sensitivity profile of the 11 isolates was studied by VITEK®2 system as depicted in Table 2. Out of the 11 isolates, the strain *S. maltophilia* was found to exhibit maximum resistance (resistant against 17 antibiotics), followed by *M. morganii* (resistant against 7 antibiotics), strains of *E. cloacae* (resistant against 4–5 antibiotics), while *K. intermedia* and *C. freundii* were observed to show minimum resistance (resistant against 1 antibiotic each) (Figure 3).

Six isolates were found to be resistant against the beta-lactam/beta-lactamase inhibitor antibiotic combination amoxycillin-clavulanic acid (Figure 4). Significant numbers of isolates were found to be resistant against ampicillin, cefuroxime and cefuroxime axetil whereas some resistance was observed against piperacillin-tazobactam. In Gram-negative bacteria, the resistance to beta-lactam antibiotics is mainly conferred by the expression of beta-lactamase genes which inactivate the beta-lactam ring like *bla* genes and *ampC* (Zhang *et al.* 2009). Interestingly, as many as five isolates were also found to be resistant against last resort polypeptide antibiotic, colistin, used to treat enterobacteriaceae infections. Resistance to colistin can be intrinsic or acquired. The strategies employed to resist these cationic polypeptide antibiotics include alteration of lipopolysaccharide layer, expression of efflux pumps and capsule formation (Olaitan *et al.* 2014). Among

Table 2 | Antibiotic resistance profile of the 11 isolates

		Strain										
Antibiotic	**Category of antibiotic**	***Acinetobacter baumannii*** (JPJC-S1)	***Enterobacter cloacae*** ssp. dissolvens(JPJC-S2)	***Citrobacterfreundii*** (JPJC-S3)	***Kluyvera intermedia*** (JPJC-S4)	***Morganellamorganii*** (JPJC-S6)	***Escherichia coli*** (JPJC-S7)	***Escherichia coli*** (JPJC-S8)	***Klebsiella pneumoniae*** (JPJC-S9)	***Aeromonashydrophila*** (JPJC-S10)	***Enterobacter cloacae*** ssp. cloacae (JPJC-S13)	***Stenotrophomonas maltophilia*** (JPJC-C)
Ampicillin	β lactam-penicillin	ND	ND	ND	R	R	S	S	R	ND	ND	R
Amoxicillin-Clavulanic acid	β lactam-penicillin/β lactamase inhibitor	R	R	ND	S	R	S	S	R	I	R	R
Piperacillin/Tazobactam	β lactam-penicillin/β lactamase inhibitor	S	S	S	S	S	S	S	S	R	S	R
Cefuroxime	β lactam-cephalosporin-2nd generation	R	R	ND	S	R	S	S	S	S	R*	R
Cefuroxime Axetil	β lactam-cephalosporin-2nd generation	R	R	ND	S	R	S	S	S	ND	R*	R
Ceftriaxone	β lactam-cephalosporin-3rd generation	S	S	S	S	S	S	S	S	ND	S	R
Cefoperazone/Sulbactam	β lactam-cephalosporin-3rd generation/β lactamase inhibitor	S	S	S	S	S	S	S	S	S	S	R
Cefepime	β lactam-cephalosporin-4th generation	S	S	S	S	S	S	S	S	S	S	R
Ertapenem	β lactam-carbapenems	S	S	ND	S	S	S	S	S	ND	S	R
Imipenem	β lactam-carbapenems	S	S	S	S	S	S	S	S	S	S	R
Meropenem	β lactam-carbapenems	S	S	S	S	S	S	S	S	ND	S	R
Amikacin	Aminoglycoside	S	S	ND	S	S	S	S	S	S	S	R
Gentamicin	Aminoglycoside	S	S	S	S	S	S	S	S	S	S	R
Nalidixic acid	Quinolone	S	S	ND	S	S	S	R	S	ND	S	R
Ciprofloxacin	2nd generation fluoroquinolone	S	S	S	S	S	S	S	S	S	S	S
Tigecyclin	3rd generation tetracycline derivative within a class called glycylcyclines	S	S	S	S	R	S	S	S	S	S	S
Nitrofurantoin	Nitrofuran	S	S	ND	S	R	S	S	S	ND	I	R
Colistin	Polypeptides	S	R	R	S	R	S	S	S	S	R	R
Trimethoprim/Sulfamethoxazole	Diaminopyrimidines	S	S	S	S	S	S	S	S	S	S	R

R, resistant; S, sensitive; I, intermediate; ND, not determined for the respective bacterium on the VITEK®2 standard AST-N280 card due to clinical irrelevance of the antibiotic according to CLSI guidelines; R*, resistant according to the Advanced Expert system™ (AES) modified MIC values as per CLSI guidelines for the respective bacterium.

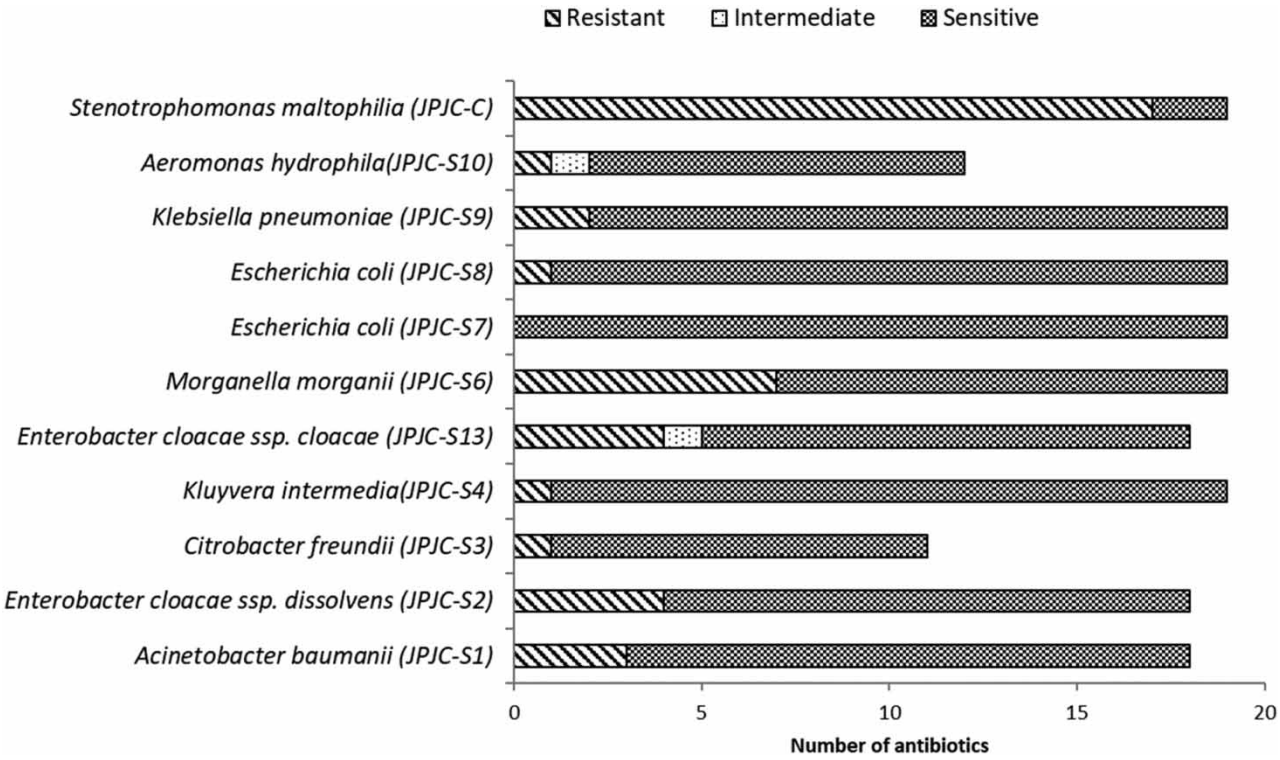


Figure 3 | Antibiotic sensitivity profile of GNBC isolated from municipal treated effluent.

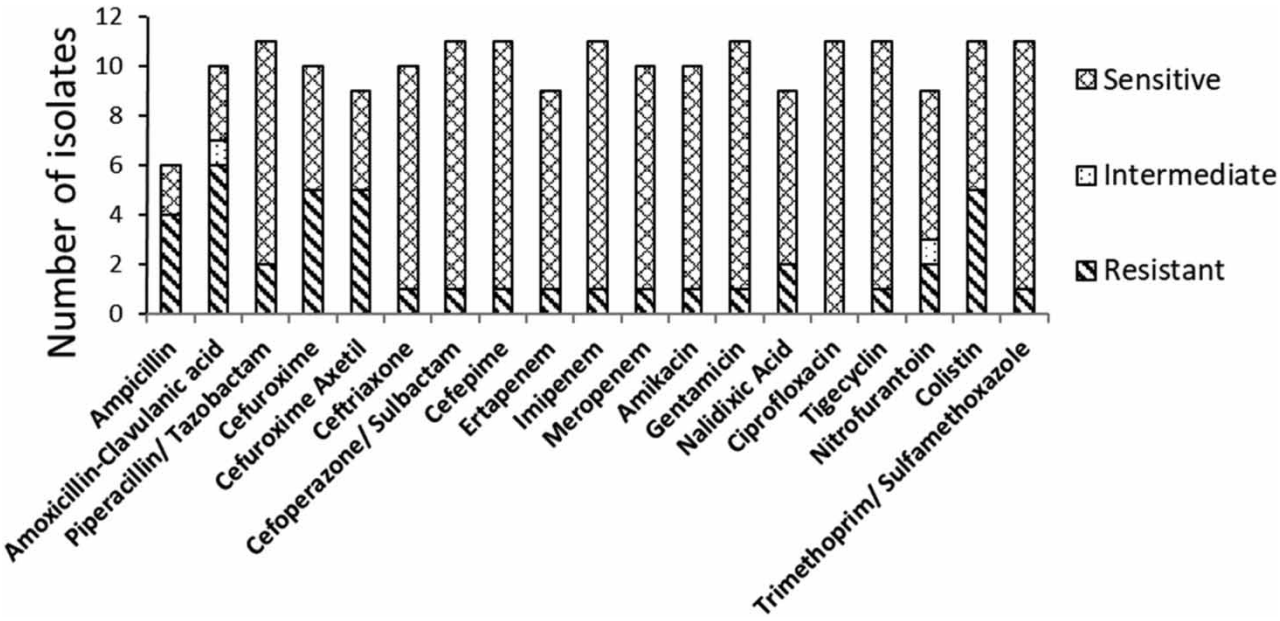


Figure 4 | Resistance against different antibiotics observed in the GNBC isolates.

the two strains each one was found to be resistant against nalidixic acid and nitrofurantoin. Resistance to nalidixic acid can be achieved by modification of DNA gyrase and topoisomerase enzymes, expression of multidrug efflux pumps or protection of DNA gyrase by Qnr protein (Ruiz 2003). Similarly, resistance to nitrofurantoin may arise via mutation

in oxygen-insensitive reductases that prevents conversion of nitrofurantoin molecule into toxic intermediate or via expression of plasmid-mediated efflux genes (Osei Sekyere 2018). None of the isolates except *S. maltophilia* showed resistance against aminoglycosides (amikacin and gentamicin), sulfamethoxazole/trimethoprim (SMX-TMP), carbapenems (ertapenem, imipenem, meropenem), third generation cephalosporin (ceftriaxone), third generation celphalosporin/beta-lactamase inhibitor combination (cefoperazone-sulbactam) and fourth generation cephalosporin (cefepime). The resistance to a large number of antibiotics observed in *S. maltophilia* may be due to the presence of intrinsic as well as acquired resistance factors such as multidrug efflux pumps, reduced membrane permeability, antibiotic modifying enzymes and the expression of quinolone resistance gene Smqnr (Sánchez 2015).

There is a significant variation in antibiotic resistance among observed isolates compared to that reported in the literature (Jacobs *et al.* 2017). Different strains of the same species may exhibit varying antibiotic susceptibilities due to differences in their genetic composition. These differences may arise due to the different environmental settings as well as the specific antibiotic preferences in different geographical locations. A metagenomics study reported from sediments collected from Mutha River, Pune, India which receives treated and untreated sewage from Pune city has found a strong correlation between the presence of *Acinetobacter* sp. and the presence of carbapenemase gene in the samples signifying carbapenem resistance (Marathe *et al.* 2017). *A. baumannii* strain isolated in this study was found to be sensitive against carbapenems but resistant to amoxicillin-clavulanic acid, cefuroxime and cefuroxime-axetil. The loss of intrinsic resistance against ertapenem may be explained by reversion to susceptible phenotype (Koskella 2018). The spread of antibiotic-resistant *A. baumannii* through hospital wastewater is not clearly understood. In a study carried out by Zhang *et al.* (2013) for the presence of *A. baumannii* strains in hospital sewage in Beijing, China, the drug resistance pattern of isolates derived from hospital sewage samples was found to be distinct from the clinical isolates. Whereas, in another study by Music *et al.* (2017) on the role of environmental reservoirs of *A. baumannii* in hospital outbreaks related to extremely drug-resistant variant, a high correlation of environmental and clinical isolates has been reported suggesting emergence of *A. baumannii* through inefficiently treated hospital wastewater. *M. morganii* strain isolated in the present study was found to be the second most resistant among the 11 isolates. It was found to be resistant against ampicillin, amoxicillin/clavulanic acid, cefuroxime, cefuroxime axetil, tigecyclin, nitrofurantoin and colistin. However, no resistance was observed against nalidixic acid and ciprofloxacin. *M. morganii* is intrinsically resistant to ampicillin, amoxicillin-clavulanic acid, colistin and nitrofurantoin but susceptible to tigecyclin (EUCAST 2016). The presence of tigecyclin-resistant *M. morganii* was observed in this study, that raises grave concerns regarding the emergence of antibiotic resistance against third generation antibiotics. *C. freundii* strain isolated in the present study was found to be resistant only against colistin. Acquired colistin resistance due to the acquisition of resistance genes has been reported in this bacterium (Li *et al.* 2017). In the present study, *Klebsiella* sp. was detected to be resistant against beta-lactam antibiotics, ampicillin and amoxicillin-clavulanic acid. The antibiotic sensitivity profile of *Klebsiella* sp. isolated from untreated hospital waste from Dhaka, Bangladesh has revealed resistance against ampicillin and suggested wide prevalence of the ampicillin resistance gene in this bacterium (Rabbani *et al.* 2017). *S. maltophilia* strain isolated in the present study was found to be resistant against almost all antibiotics including SMX-TMP and was found to be sensitive to second generation fluoroquinolone ciprofloxacin. Increasing incidences of SMX-TMP resistance are being reported in clinical isolates (Kumar *et al.* 2020). *S. maltophilia* is an opportunistic pathogen and is resistant to various antibiotics (Furlan *et al.* 2019; Kumar *et al.* 2020). The presence of this uncommon bacterium in domestic wastewater raises the possibility of hospital wastewater intrusion in the domestic sewage. Recently, a novel *S. maltophilia* strain, a clinical isolate, was reported to possess antimicrobial resistance genes as well as biocide and heavy metal resistance genes (Kumar *et al.* 2020).

Aeromonas sp. was observed to be resistant against multiple antibiotics that support the dissemination of antibiotic resistance among bacterial community in wastewater (Igbinosa & Okoh 2012). The resistance profile of *A. hydrophila* isolate in this study was found to be similar to that of clinical isolates as studied by Vila *et al.* (2002). The authors reported all *A. hydrophila* strains to be susceptible to ciprofloxacin, cefuroxime, cefepime, whereas 90% of strains were susceptible to SMX-TMP. However, 100% susceptibility to piperacillin/tazobactam has also been reported in Vila *et al.* (2002), which does not match our observations. The observed variations in resistance pattern might be because of the differences in strain characteristics due to difference in environmental conditions and antibiotic preferences in different regions. In this study, *K. intermedia* was found to be resistant only against ampicillin and not against other beta-lactam antibiotics. Intrinsic resistance for beta-lactam antibiotics has been reported for this bacterium (EUCAST 2016). The survival of MDR Gram-

negative bacteria predominantly in treated effluent is a serious concern for public health safety. Therefore, wastewater treatment technologies must be implemented to limit the antibiotic-resistant bacteria in treated effluent.

3.3. Screening of isolates for ESBL activity and molecular identification of ESBL gene

Five isolates, namely, *A. baumannii*, *E. cloacae* ssp. dissolvens, *E. cloacae* ssp. cloacae, *S. maltophilia* and *M. morganii* were observed to be resistant against multiple beta-lactam antibiotics upon VITEK®2 analysis (Table 2). Although ESBL producing *blaCTX-M* gene has been reported to be naturally present in *Kluyvera* species, the *Kluyvera* strain reported in this study was not tagged as ESBL positive in VITEK®2 analysis. The strains suggested to be ESBL producers in VITEK®2 analysis were screened by phenotypic confirmatory disc diffusion test for ESBL production and out of the five isolates, two (*E. cloacae* ssp. dissolvens and *S. maltophilia*) were found to be positive for ESBL production.

The ESBL producer strains confirmed by phenotypic disc diffusion test were genotypically characterized for the gene responsible for ESBL activity. In both of the isolates, only *blaCTX-M* gene was detected whereas *blaTEM* and *blaSHV* genes were found to be absent (Figure 5). A high prevalence of *blaCTX-M* gene has been reported in ESBL producing Gram-negative bacteria (Zeynudin *et al.* 2018). In fact, *blaCTX*-M gene has been reported to be the most prevalent ESBL gene in non-fermenting Gram-negative bacterial isolates (Gupta *et al.* 2016). Both of the *blaCTX-M* positive isolates observed in this study (*S. maltophilia* and *E. cloacae*) are non-lactose fermenting. *E. cloacae* is an important environmental bacterium that may also act as an opportunistic pathogen and it is known to be a common producer of ESBL (Chagas *et al.* 2011). One of the two strains of *E. cloacae* isolated in this study was found to harbour *blaCTX-M* gene whereas *blaTEM* and *blaSHV* genes were found to be absent in both the strains. In a study carried out by Chagas *et al.* (2011) on predominance of ESBL genes in *E. cloacae* isolates in treated hospital wastewater, *blaCTX-M* was detected in a majority of isolates and this gene was found to co-occur with *blaTEM* genes. However, in our study, only *blaCTX-M* was detected and none of the isolates were found to be positive for *blaTEM* and *blaSHV*. Thus, no direct correlation could be established between clinical isolates and *E. cloacae* strains isolated in this study on the basis of the presence of ESBL genes. Moreover, both the *E. cloacae* strains in this study were found to be resistant to colistin which is a serious concern in the light of the fact that colistin is a last resort drug. However, the molecular basis of colistin resistance could not be established as ARGs related to colistin resistance were not analysed in this study.

S. maltophilia strain isolated in this study was also found to harbour *blaCTX-M* gene. Although *blaCTX-M* gene has been detected in a few *S. maltophilia* isolates, the ESBL production in this bacterium is more frequently linked to chromosomal genes L1 and L2, which are responsible for its intrinsic resistance to beta-lactams (Okazaki & Avison 2008). The *blaSHV* was found to be the most prevalent ESBL gene in *S. maltophilia* strain identified from fish farm (Adelowo *et al.* 2018), again highlighting the fact that different strains of the same species may differ in their antibitotic resistance characteristics. *S. maltophilia* is intrinsically MDR to many antibiotics. It has been reported that environmentally isolated *S. maltophilia* harbours many acquired antibiotic resistance genes (Furlan *et al.* 2019). Therefore, the antibiotic resistance profile of such bacteria limits the therapeutic options, which is a serious concern for public health safety. It has been reported that *blaCTX-M* gene can be associated with a variety of mobile genetic elements that mediate the rapid mobilization to yield

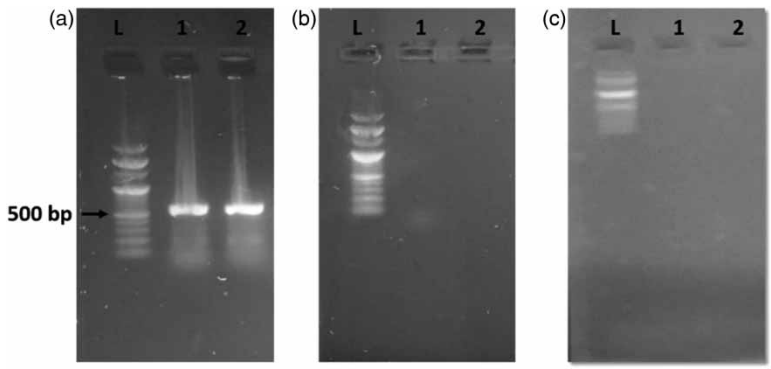


Figure 5 | Detection of gene encoding *blaCTX-M* (a), *blaSHV* (b) and *blaTEM* (c) in ESBL-producing isolates: 1 (*E. cloacae* ssp. cloacae) and 2 (*S. maltophilia*). L, 100 bp ladder.

multi-resistant clones (D'Andrea *et al.* 2013). Therefore, the presence of MDR bacteria associated with *blaCTX-M* gene is alarming (Zeynudin *et al.* 2018).

The confirmation of such high antibiotic resistance forces us to rethink the existing wastewater reuse norms, which is entirely based on coliform standards. Most of the disinfection methods have sensitivity against specific antibiotics, antibiotic-resistant bacteria and antibiotic resistant genes (Shekhawat *et al.* 2020b). Therefore, the development of a suitable disinfection strategy is needed to tackle the reductions in these emerging pollutants along with the removal of coliforms.

4. CONCLUSIONS

The presence of multiple drug resistance in most dominant Gram-negative isolates from treated sewage is alarming. The isolates were observed to be resistant against the antibiotics amoxicillin-clavulanic acid, cefuroxime, cefuroxime axetil and a last resort drug, colistin. *blaCTX-M* encoded ESBL activity was confirmed in two of the isolates. This study suggests a presumptive method for detection of the prevalence of antibiotic resistance in wastewater, through dominantly occurring bacterial isolates. Although focusing on a subsection of the bacterial population may lead to loss of some valuable data on less prevalent species, such studies could help in the rapid investigation of resistance prevalence in sewage and help towards framing a policy for its reuse. Further, a suitable disinfection strategy may be designed for public health and the environment safety. Thus, an integrative study is recommended for further implementation of the findings from this work.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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