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Surveillance of two Noida drains for assessing the presence of carbapenem-resistant ESKAPE bacteria

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***Abstract*---**Antimicrobial resistance (AMR) is a major public health concern for clinicians all over the world. It occurs due to careless consumption of antibiotics and not completing the prescribed regimen. In India, environmental factors such as pollution, lack of cleanliness and hygiene also exacerbate the problem. If efforts are not put into combating this threat it is predicted to have a devastating impact on the economy and will be a deterrent in achieving United Nation's sustainable development goals. Regional and periodical characterization of microbial resistome is essential for timely treatment decisions by doctors and prevention of the spread of drug-resistant infections. However, obtaining AMR data from clinical

infections requires ethical clearances, and may not even provide a complete and accurate spectrum of all relevant AMR genes (as microbiota of healthy persons may have different AMR genes). Since both domestic and industrial waste contributes to the spread and development of antimicrobial resistance, identification of multidrug-resistant strains in untreated sewage (that provides testing samples from mostly healthy population) for surveillance and prediction of AMR may be more reliable, ethically acceptable and economically feasible approach. The current study involves collecting sewage samples from Noida drains and isolating meropenem resistant colonies by streaking on Mueller Hinton Agar supplemented with 5µg/ml of antibiotic. Subsequent characterization by employing standard morphological techniques was followed by susceptibility testing towards 11 clinically relevant antibiotics. Alarmingly 15.3% of isolates were resistant to all antibiotics and 46.2% were resistant to commonly used antibiotics such as Ampicillin. Based on 16s rRNA gene sequencing of short listed 6 MDR isolates found to be resistant to almost all antibiotics tested, 4 of them match with *Klebsiella sp* and 2 with *Enterococcus sp*. The study identifies a high level of MDR strains in Noida drains and urges measures for preventing their transfer to pathogenic organisms that infect humans or animals.

***Keywords*---**Carbapenem-Resistant, Antimicrobial resistance, Sewage, Antimicrobial susceptibility, Kirby-Bauer disk diffusion.

1. Introduction

Antimicrobial resistance (AMR) refers to the ability of microbes to resist killing by antimicrobials (antibiotics) for their survival due to the gain of antibiotic-resistant genes (ARGs). The microbe exhibiting resistance may be further classified as multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) depending on its resistance to at least one drug in three or more antimicrobial categories, to most of the available antimicrobial categories barring one or two and to all antimicrobial categories respectively (Magiorakos et al., 2012). Although, the evolution of mechanisms of resistance is a natural phenomenon, however, since the last few decades owing to overuse and misuse of antimicrobials, AMR has grown at an alarming rate and is estimated to be the leading cause of death globally, even higher than AIDS or Malaria (Abbafati et al., 2020; Murray et al., 2022). Additionally, it affects the economic status of every country because long treatment schedules and expensive medications increase the cost of treatment (Papp-Wallace et al., 2011). According to various reports this cost is calculated to be $105 billion, or US $28.4 trillion annually (Nordmann & Poirel, 2019). WHO has announced AMR as an urgent priority and since it is a muti-faceted problem suggested one health approach (taking care of human health, animal health and environment) to combat the problem. With microbes acquiring resistance to almost all antibiotics, especially the last resort carbapenem, looking at the grim situation, 68th World health Assembly proposed multi-pronged Global Action Plan to contain AMR (GAPAMR) in May 2015 that included developing new therapeutics and diagnostics, generating awareness and

understanding of AMR through education and training, reducing infection through improved hygiene and sanitation, framing regulations for appropriate use of antibiotics and constant surveillance of AMR bugs (World Health Organization, 2015).

India carries the largest burden of drug resistant microbes that are resistant even to the newly discovered antibiotics. Amongst the Gram negatives, greater than 70% isolates of *Escherichia coli, Klebsiella pneumoniae* and *Acinetobacter baumannii* are found resistant to fluoroquinolones and third generation cephalosporins. In fact 65% of *K. pneumoniae* is resistant due to presence of carbapenem degrading carbapenemases (*Scoping Report on Antimicrobial Resistance in India - Center for Disease Dynamics, Economics & Policy (CDDEP)*, n.d.). It is observed that the mortality rates in patients infected with carbapenem-resistant *Enterobacteriaceae* is higher as opposed to those infected with susceptible strains (Mauldin et al., 2010). Emergence of NDM (New Delhi metallo-β-lactamases) named after the capital of India where it was detected first, is rapidly spreading to other countries (Dixit et al., 2019). Alarming rates of resistance have been reported in animal isolates of human pathogens, but the evidence is insufficient to make national estimates. About 29% of isolates of *Staphylococcus* species were methicillin resistant in 2008, but increased to 47% in 2014. Because of active research and highly active committees the numbers are declining every year for other countries but not so for India. Some environment specific factors that could contribute to development of AMR are (1) Contamination of water bodies with pharmaceutical waste and hospital effluents due to inadequate treatment and lack of stringent regulations (2) Overuse of antimicrobial agents for health of food producing animals and agriculture industry, and (3) Socio economic and cultural habits prevalent in India such as defecating in open areas, resulting in seeping of antibiotic residuals into soil /water or in high antibiotic concentrations in municipal wastewater which if not effectively treated will be reservoir for breeding of resistant organisms (Taneja & Sharma, 2019). However, scarcity of data and research evidence not only impedes the exact estimation but also hampers the efforts to tackle this problem and puts India in a great danger of succumbing to AMR mortality in coming few years. Uttar Pradesh is the most populated state of India and Noida specially is part of NCR region where people from all over India migrate and reside for living. No AMR surveillance from Noida region has been reported so far and present study aims to evaluate if multiple drug resistance bacteria is present in sewage sources in Noida, especially clinically relevant carbapenem resistant ESKAPE members. Sewage contains waste from houses, industries and hospitals and comprises a wide variety of microbes and therefore a sample from single site can represent large population for which otherwise it is difficult to gather data. Sewage sampling is an affordable surveillance option, easy to implement in resource-limited settings. With this backdrop, the focus of the current study was to collect resistance data from Noida sewages and to postulate likely AMR trends in the region. The work provides unambiguous evidence for prevalence of multidrug resistant *Klebsiella pneumoniae* and *Enterococcus faecium* in sewage sample collected from representative drains of Sector 51 and Sector 72 of Noida region. This has implications for forming intervention strategies in the region for public health.

2. Materials & Methods

2.1 Sample Collection

Two wastewater samples were collected on the day 15th of March 2022 from Noida Sector 51 (Site 1) and Noida Sector 72 (Site 2). Although these sites were selected randomly but after searching the nearby areas these sites were found to carry the effluents from the nearby hospitals namely Manas Hospital (S1), Cloud 9 (S1), Fortis Hospital (S1) and Sai Hospital (Site 2). The samples were collected at depth of 25 cm in a sterilized container and labelled as Site 1 (S1) and Site 2 (S2) and stored at 4 °C for further experiments.

2.2 Sample Processing and Isolation of Bacteria

The samples were transported to the laboratory in refrigerated conditions for further processing within 2 hours of sample collection. These samples were serial diluted to 10^{-4} dilutions and 100µl from each sample were plated on Mueller Hinton Agar (MHA) supplemented with a 5µg/ml of Meropenem for the primary selection of carbapenem-resistant bacteria. The plates were incubated at 37 °C for 24 hours and based on colony morphology, a total of 14 colonies were picked from both the samples and labelled as isolates 1-14 (I1-I14). These colonies were further streaked on nutrient agar plates in order to get isolated and pure colonies. Due to the slow growth of I10, it was not considered for further experiments.

2.3 Antibiotic Susceptibility Test

The Kirby-Bauer disc diffusion method [10] was used to test the antibiotic susceptibility of 13 isolates against 11 antibiotics namely Azithromycin (15µg/ml), Ciprofloxacin (5µg/ml), Levofloxacin (5µg/ml), Amikacin (30µg/ml), Cefuroxime (30µg/ml), Ampicillin-Sublactam (10/10 µg/ml), Ampicillin (10µg/ml), Aztreonam (30µg/ml), Cefotaxime (30µg/ml), Amoxyclay (30µg/ml), and Imipenem (10µg/ml). For antibiotic susceptibility, the isolates were cultured in nutrient broth till the O.D. at 625 nm was 0.8. 500µl of each culture was plated on MHA and the plates were incubated at 37 °C for 16 hours. The diameter for the zone of inhibition was then measured for each isolate and compared with CLSI (formerly NCCLS) & EUCAST standards.

2.4 Characterization and identification of isolated MDRs

The isolates showing resistance against more than 5 antibiotics were selected and characterized by gram staining using standard procedure. Briefly, the culture was taken from the plates and spread on the glass slide to make a smear in a drop of 0.9% saline followed by heat fixing of the smear. The smear was stained with primary stain crystal violet for 1 minute and then the gram's iodine was applied for another one minute. Destaining of the smear was done by washing it with 95% ethanol. The counter stain safranin was used for further staining of the bacterial cells for a minute. The slide was rinsed with water and viewed under 100X with emulsion oil.

2.5 Identification of MDR isolates by 16s rRNA sequencing

The identification of MDR isolates was carried out by 16s rRNA sequencing which was outsourced through BioKart India Pvt Ltd. (Karnataka, India).

3. Results and Discussion

Figure.1 shows the sites in Noida city (which is part of the National Capital Region of India) from where the sewage samples were collected. The two sectors 51 and 72 are marked blue in the Figure. 1, carry effluents from communities as well as hospitals (marked red in Fig. 1). Following collection, the samples were transported to the laboratory in a cooler box containing ice packs for further analysis.

Isolation of pure Carbapenem-resistant colonies from sewage samples

For the primary selection of carbapenem-resistant bacteria and to obtain pure colonies, 5µg/ml of Meropenem (a member of carbapenem class of antibiotics and a broad spectrum antibiotic) was added to the agar plates. This concentration is higher than the interpretive standards for meropenem susceptibility standard (as defined by CLSI and EUCAST) corresponding to MIC of ≤4 µg/ml, since literature reports wide range of variation in MICs of carbapenem resistant strains. Subsequently, after obtaining the pure colonies on meropenem, they were tested on 11 additional antibiotics (from different classes) through the disc diffusion assay. Results of the same are depicted in Table 1, where the range for Resistant (R), Intermediate (I) and Sensitive (S) as per CLSI interpretive standards, for each isolates is also given in the column adjacent to the reference antibiotic. Most of the isolates exhibit resistance to more than 1 antibiotic tested (77%), only I2, I5 and I6 were susceptible (23%) to all antibiotics tested (Table 1). Two isolates (15.3%) namely, I3 and I12 were resistant against all the tested antibiotics. 46.2% isolates (I1, I3, I4, I7, I8 and I12) are resistant towards multiple antibiotics (MDRs). Interestingly, I1 and I4 exhibit resistance against antibiotics belonging to other than carbapenem group and are susceptible to imipenem.

Characterization and Identification of MDR isolates

The 6 MDR isolates I1, I3, I4, I7, I8 and I12 were further characterized by gram staining. I1, I3, I4 and I10 were gram-negative rods whereas I7 and I8 were found to be gram-positive cocci (Table 2). Based on the BLAST search of the 16s rRNA sequenced data (Table 3), isolates collected from Sector 72, Noida (I1, I3 and I4) are identified belonging to *Klebsiella sp.* whereas those collected from Sector 51 (I7 and I8) match with *Enterococcus sp.* Surprisingly, no drug resistant *E. coli* was isolated in this study and the reason could be that primary screening entailed 5µg/ml of meropenem. AMR annual report of 2020 by Indian Council of Medical Research (*AMR ICMR Data*, n.d.) reports 76% *E. coli* to be susceptible towards carbapenem. It is possible that the ubiquitous *E. coli* is susceptible to Meropenem and hence not isolated in current study as this antibiotic was used for primary selection of drug resistant bacteria in a concentration which was higher than its MIC of ≤4 µg/ml. This suggests that the hospital effluent which is expected to harbour clinical carbapenem resistant *E.coli* is not influencing the overall

bacterial diversity and carbapenemase-encoding genes pool in wastewaters of reference sites. Overall, this study provides evidence that *Klebsiella pneumoniae* and *Enterococcus sp.* are the MDR organisms present in Noida sewages and strategies need to be established for effective treatment of wastewater to mitigate their dissemination into the environment and spread in communities.

4. Conclusion

The existence and spread of carbapenem-resistant bacteria is generally associated with health care facilities but recently these bacteria are increasingly being disseminating in communities through air and water bodies. Hence, a key resource for AMR surveillance is sewage drains that holds antibiotic resistance microbes. This study is an attempt to catalogue carbapenem resistant microbes present in sewage of Noida, NCR and provides evidence of existence of highly drug resistant *Klebsiella pneumoniae* and *Enterococcus faecium*. In addition, as different drug resistant profile is observed from two sites, it's probably reflecting antibiotic usage in that area. These findings suggest close monitoring of the epidemiology of these strains and the need to maintain a judicious antibiotic use policy. Whole genome sequencing will further provide knowledge of ARGs and resistance mechanisms which will help in designing strategies to reduce burden of AMR.

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Table 1: Antibiotic Susceptibility Results of Bacterial Isolates from Sewage Samples

S. No.	Antibiotic Disk (Dose in µg/disc)	Antibiotic Class	Standard interpretation as per CLSI & EUCAST (mm)			Isolate Id												
			R	I	S	JIIT/ NS72 /I1	JIIT/ NS72 /I2	JIIT/ NS72 /I3	JIIT/ NS72 /I4	JIIT/ NS72 /I5	JIIT/ NS51 /I6	JIIT/ NS51 /I7	JIIT/ NS51 /I8	JIIT/ NS51 /I9	JIIT/ NS51 /I11	JIIT/ NS51 /I12	JIIT/ NS51 /I13	JIIT/ NS51 /I14
1	Cefuroxime (30)	Cephalosporin	≤14	15 -17	≥18	R	S	R	R	S	S	R	R	R	R	R	R	R
2	Aztreonam (30)	Monobactam	≤15	16 - 22	≥22	R	S	R	R	S	S	R	R	R	R	R	R	R
3	Azithromycin (15)	Macrolide	≤13	14 - 17	≥18	R	S	R	R	S	S	R	R	I	R	R	I	I
4	Levofloxacin (5)	Quinolone	≤13	14 - 16	≥17	R	S	R	R	S	S	R	R	S	S	R	S	S
5	Amikacin (30)	Aminoglycoside	≤16	17 -63	≥64	R	S	R	R	S	S	R	R	I	R	R	I	I
6	Ampicillin (10)	Penicillins	≤8	9 -31	≥32	R	S	R	R	S	S	R	R	R	I	R	R	R
7	Ciprofloxacin (5)	Quinolone	≤15	16 -20	≥21	R	S	R	R	S	S	R	R	S	I	R	S	S
8	Imipenem (10)	Carbapenem	≤16	17- 18	≥19	S	S	R	I	S	S	R	R	S	S	R	S	S
9	Amoxyclav (30)	Penicillin, beta-lactamase inhibitor	≤2	3 -7	≥8	R	S	R	R	S	S	S	S	S	S	R	S	I
10	Ampicillin/ Sublactam (10/10)	Penicillin, beta-lactamase inhibitor	≤11	12 -14	≥15	R	S	R	R	S	S	R	R	R	S	R	R	R
11	Cefotaxime (30)	Cephalosporin	≤14	15 - 22	≥23	R	S	R	R	S	S	R	R	R	I	R	R	R

Table 2: Morphological identification of selected isolates

S. No.	Isolates	Gram Character	Cell morphology and arrangement
1	JIIT/NS72/I1	Negative	Small rods in short chain
2	JIIT/NS72/I3	Negative	Small rods in short chain
3	JIIT/NS72/I4	Negative	Small rods in short chain
4	JIIT/NS51/I7	Positive	Cocci in pairs
5	JIIT/NS51/I8	Positive	Cocci in pairs
6	JIIT/NS51/I12	Negative	Small rods in short chain

Table 3: Results of 16s RNA Sequencing

Isolate ID (query)	NCBI BLAST			Genera & species of the isolate
	Closest Match	GenBank Accession ID	% Identity to closest match	
JIIT/NS72/I1	*Klebsiella pneumoniae* strain SCPM-O-B-8922 (20PKP/19c) chromosome	CP094363.1	100	*Klebsiella pneumoniae*
JIIT/NS72/I3	*Klebsiella pneumoniae* subsp. ozaenae strain AM17 16S ribosomal RNA gene	ON323049.1	99.86	*Klebsiella pneumoniae*
JIIT/NS72/I4	*Klebsiella sp. strain* InAD-061 16S ribosomal RNA gene	MF401267.1	99.86	*Klebsiella sp*
JIIT/NS51/I7	*Enterococcus faecium* strain R-5 16S ribosomal RNA gene	ON112104.1	100	*Enterococcus faecium*
JIIT/NS51/I8	*Enterococcus* sp. strain B5C 16S ribosomal RNA gene	ON318408.1	100	*Enterococcus* sp.
JIIT/NS51/I10	*Klebsiella* sp. NCCP-141 gene for 16S rRNA	AB558500.1	100	*Klebsiella* sp.

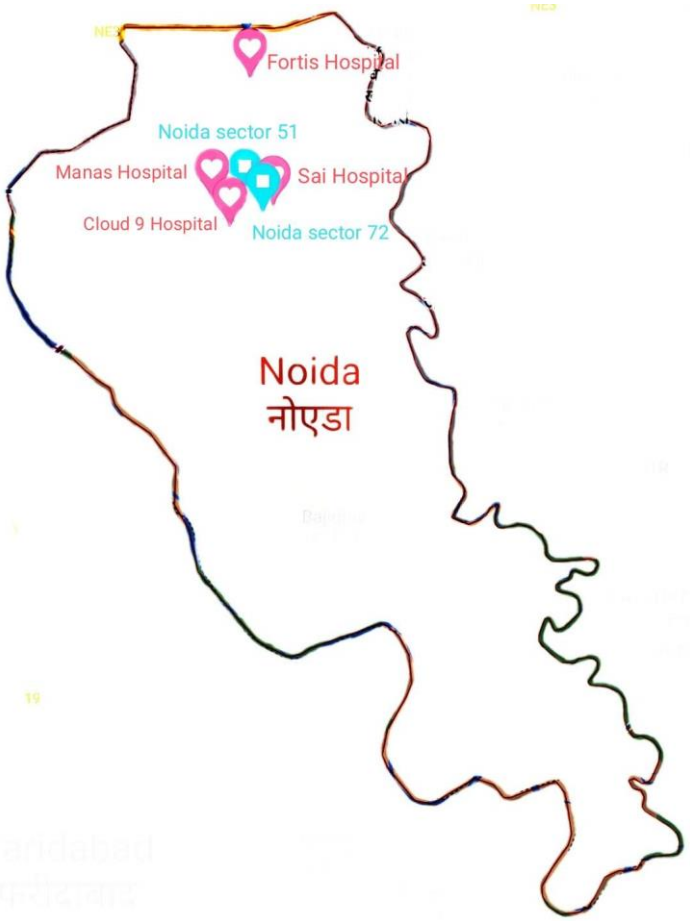

Figure 1: Map of Noida region representing the sample collection sites (sector 51 and 72) and the hospitals in the vicinity of the sites.