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Hospital wastewater releases of carbapenem-resistance pathogens and genes in urban India

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ABSTRACT

Increasing antibiotic resistant hospital-acquired infections and limited new antibiotic discovery are jeopardizing human health at global scales, although how hospitals themselves fuel AMR in the wider environment is largely unknown. Antibiotic resistance (AR) from hospitals in countries like India is potentially problematic because of high antibiotic use, overcrowding, and inadequate wastewater containment. Here we quantified fecal coliforms (FC), carbapenem-resistant Enterobacteriaceae (CRE), blaNDM-1, and selected extended-spectrum β-lactam (ESBL) resistant bacteria and genes in 12 hospital wastewater outfalls and five background sewer drains across New Delhi over two seasons. Hospital wastewaters had up to nine orders of magnitude greater concentrations of CRE bacteria and blaNDM-1 than local sewers (depending on the hospital), implying hospitals contribute high concentrations of AR relative to community sources in Delhi, especially during the winter. Significant correlations were found between FC levels (a fecal indicator), and CRE (r =0.924; p =0.005), blaNDM-1 (r=0.934, p=0.009), and ESBL-resistant bacteria (r=0.913, p=0.010) levels across hospital wastewaters, respectively, implying elevated CRE and blaNDM-1 are of patient origin. However, of greater importance to global health, microbial culturing found 18 to 41% of wastewater CRE isolates (n =1447) were on the WHO “critical pathogen” list in urgent need of new antibiotics, and 55% of CRE isolates from larger hospitals carried at least one blaNDM-1 gene. Wastewater releases from New Delhi hospitals may pose a greater AR exposure risk to residents than believed, implying in-hospital antibiotic use must be better controlled and more effective waste treatment is needed for hospital wastewaters.
**Introduction**

Antibiotic resistance (AR) is a global public health concern. In fact, emergence of resistant and untreatable phenotypes in hospital-acquired infections and limited new antibiotic discovery is threatening return to a pre-antibiotic era\(^1,2\). Wastewater releases from hospitals contribute to this problem. Antibiotics are routinely used to treat infections, but treatment results in antibiotic residues, and resistant bacteria (ARB) and genes (ARGs) being excreted in patient wastes, entering hospital wastewater systems\(^3\). Waste-born nosocomial and commensal ARB carrying ARGs are then released to the wider environment into sewers, exposing individuals away from hospitals to AR of hospital origination. Although many gut flora die-off upon release to sewers, they can exchange ARGs to environmental strains via horizontal gene transfer (HGT) on plasmids, transposons and integrons\(^4,5\). As such, hospital antibiotic use can lead to wider ARB and ARG dissemination via wastewater, becoming a potential health concern, especially in parts of world with inadequate wastewater treatment and community sanitation.

In emerging countries, such as India, this problem is particularly acute. Local domestic wastewater treatment is often not adequate, and clean, safe water is not assured\(^6\). As an example, only \(~40\%\) of wastewater in New Delhi is treated\(^7,8\); therefore, ARB and ARGs released in hospital wastes to community sewer drains pose a particular exposure risk due to the inadequate urban infrastructure. This is especially key relative to hospitals in Delhi because antibiotic use is often high; overcrowding is common\(^9,10\); and reliable internal wastewater management is often lacking. As such, hospital wastewater releases may particularly contribute to AR spread and exposures in places like India, especially to the very poor who frequently reside near open drains. For the above reasons, AR infections are more common, causing higher per patient treatment costs, prolonged morbidity, and increased mortality rates\(^9–13\) due to nosocomial
transmission of multidrug-resistant (MDR) organisms, including *Streptococcus pneumoniae*\(^{14}\), *Mycobacterium tuberculosis*\(^{15}\), *Salmonella* sp., *Shigella*\(^{16}\), and *Vibrio cholera*\(^{17}\). ARB and ARGs released in hospital wastes pose a huge challenge in the emerging world\(^{18}\) as was suggested by the World Health Organization\(^{19}\) and other recent reports\(^{20}\).

India is the largest consumer of antibiotics for human use in the world\(^{10}\), and β-lactam antibiotics are among the most common drugs used in India, including carbapenems\(^{21}\). It is not surprising, therefore, first detection of *bla*\(_{\text{NDM-1}}\) was in a *Klebsiella pneumonia* isolate from an Indian hospital patient in 2008\(^{22}\). *bla*\(_{\text{NDM-1}}\) codes for a metallo-beta-lactamase (NDM-1) that can confer MDR\(^{23}\). After initial clinical detection, *bla*\(_{\text{NDM-1}}\) was soon found in Delhi surface waters (in 2010), and now is present in >100 countries\(^{24–26}\), including in strains functionally resistant to all antibiotics, including colistin\(^{26,27}\). However, *bla*\(_{\text{NDM-1}}\) is only one example of hospital-born AR spread via wastes. Extended spectrum β-lactamase (ESBL) *Enterobacteriaceae* were detected in Lebanese hospital wastes\(^{28}\), MDR *E. coli* in Malaysian hospital wastes (including *bla*\(_{\text{NDM-1}}\))\(^{29}\), and in Spanish hospital wastewater bacteria\(^{30}\) carrying class 1\(^{31}\) or class 3\(^{32}\) integrons

Overall, evidence show hospital wastewaters are a potentially important source of AR to the environment, especially in places like New Delhi. However, little is known about how hospital wastes impact the nature and abundance of ARB and ARGs released to the environment, especially how hospital wastewater levels compare with background levels in community sewage. To address this question, ARB/ARGs from 12 hospital waste outfalls and five sewer drains in New Delhi were quantified and characterized over two seasons. Focus here was on carbapenem-resistant Enterobacteriaceae (CRE), *bla*\(_{\text{NDM-1}}\), and selected ESBL ARGs and ARBs to guide mitigation efforts in India, although results have global implications because extreme
resistant phenotypes in places like India have the potential to spread globally via international travel and other pathways.

Materials and Methods

Study sites and samples collection: Wastewater samples were collected in triplicate from sewage outfalls to community drains from 12 hospitals in New Delhi in February/March (“winter”) and May/June (“summer”) 2014. The hospitals have been anonymised for confidentiality, but size and other data are provided in Table S1 (Supporting Information; SI), which are the best available. The hospitals were chosen based upon various criteria, including: public versus private management, bed capacity, location, and medical approaches, including allopathic and unani medicine. Most of the hospitals surveyed here are general public hospitals (government) that use both conventional and allopathic approaches in treatment. In parallel to hospital wastewater sampling, replicate wastewater samples were collected from five sewer drains in New Delhi to contrast community versus hospital sources of AR to the environment. Only drains without hospitals in their catchments were sampled, including: the Najafgarh Drain (28°31’25”N 76°54’51”E), Palam Drain (28°32’25”N 77°01’10”E), Bawana Escape Drain (28°75’44”N 77°18’28”E), Ali Drain (28°30’32”N 77°18’50”E) and Sarita Vihar Drain (28°31’30”N 77°19’12”E).

Both hospital and drain wastewater sampling was performed in the summer and winter to assess seasonal differences, such as temperature and drug use trends in each season. Specific samples were collected in sterile 500-mL containers, returned to the laboratory on ice in coolers, and stored at 4°C for further analysis. Microbial culturing was performed within 12 hours of sampling. To quantify wastewater conditions, temperature, pH, total dissolved solids (TDS) and conductivity were measured in-situ using hand-held probes (detailed in Table S2).
**Microbial Culturing of coliforms and ARB:** Microbial culturing was performed to quantify the abundance of fecal coliform (FC), total coliform (TC), ESBL-resistant bacteria (ESBL) and CRE using selective media, and to identify putative pathogens in hospital and drain wastewaters. Prior to plating, samples were serially diluted in sterile phosphate buffer solution (PBS), and then were plated at different dilutions in triplicate on M-FC Agar (FC), Rapid HiColiform Agar (TC), HiCrome ESBL Agar (ESBL ARB), HiCrome KPC Agar (to capture CRE ARB). The HiColiform, HiCrome ESBL and HiCrome KPC plates were incubated at 37˚C for 24 h, whereas M-FC plates were incubated at 45˚C for 24 h.

Phenotypically-resistant isolates from hospital wastewaters (only) were randomly selected from CRE (n = 1447) and ESBL (n = 1200) plates, and DNA extracted using the FastDNA Spin kit for Soil (MP Biomedicals, USA), according to manufacturer’s instructions. Isolated DNA was amplified using bacterial 338F and 1046R primers using conditions summarised in Table S3. Conserved 16S-rRNA gene sequences (per isolate) were analyzed using Sanger sequencing and identified by BLAST. Isolate identities were verified using Biochemical Test kits (Himedia, India). DNA from all the isolates was amplified using NDM-1-F and NDM-1-R to verify the presence or absence of \( \text{bla}_{\text{NDM-1}} \) in each isolate, using conditions summarized in Table S3.

**Quantification of selected ARGs and MGE genes using quantitative PCR (qPCR):** Samples for DNA extraction were either directly filtered through sterile 0.22-µm membrane disc filters (Pall Life Science, USA) or pelleted by centrifugation at 13,000 rpm for 30 mins. Subsequent DNA extraction employed the FastDNA Spin kit for Soils (MP Biomedicals, USA). Extracted DNA was stored at -20˚C prior to subsequent qPCR analysis. Prior to qPCR, DNA samples were
diluted (1:100) with nuclease free water to minimise background interferences of the qPCR reactions. Four ARGs (*bla*<sub>TEM</sub>, *bla*<sub>OXA</sub>, *bla*<sub>CTX</sub>, *bla*<sub>NDM-1</sub>), the integrase gene for class one integrons (*int1*), and 16S-rRNA gene were quantified using qPCR. All reactions were performed in triplicate using the BioRad CFX C1000 System (BioRad, Hercules, CA USA). All runs were performed with positive standards and negative controls. Standards probe target DNA cloned into plasmids at different dilutions, whereas negative controls were nuclease-free water. Sample preparations for each gene and programs used for qPCRs are summarised in Table S3.

**Data Analysis:** Two types of statistical tests were used; bivariate correlation analysis between individual measured parameters in wastewater samples, and two-sample tests comparing differences according to hospital size and season. For analyses here, small and large hospitals were defined by estimated patient admissions. As background, many hospitals in India admit many more patients than their listed bed capacity, including hospitals within this study. Therefore, for hospital size comparisons, the 12 hospitals were sub-divided into two groups of six with “small” hospitals having admissions of between 150 to 691 patients and large hospitals having admission from 1950 to 7270 (see SI for details).

All data analyses were performed using Excel 2007 (Microsoft Office 2007, Microsoft Corp, USA) and SPSS ver. 19.0 (IBM Inc, USA). Bivariate correlation analysis was performed using the Spearman’s non-parametric method to obtain correlation coefficients (r) and p-values. Paired sample t-tests were used to compare hospital sizes or seasons. All data were log-transformed before analysis; statistical significance was defined by 95% confidence intervals (p < 0.05).
Result and Discussion

Carbapenem- and ESBL-resistant gram-negative pathogens in hospital wastewaters are a health concern in places like New Delhi due to an insufficient urban wastewater infrastructure. Although nominal wastewater flows from hospitals are small compared to the wider community (Table S4), hospitals are major points of antibiotic use and release patient wastes containing a wide range of AR strains. Therefore, knowing and quantifying bacterial and other constituents in hospital waste streams, including AR pathogens, is critical for reducing wider dissemination and impact of hospital-originated infectious agents.

Within this context, summer and winter samples were collected from wastewater outfalls of 12 hospitals across New Delhi just prior to entry into community sewage drains. TC, FC, ESBL-resistant, and CRE abundances; selected ESBL- (bla\textsubscript{CTX}, bla\textsubscript{OXA}, bla\textsubscript{TEM}) and CRE-related (bla\textsubscript{NDM-1}) ARGs; and int1 genes (as a reflection of HGT) were quantified and sorted according to season (summarised in Tables S5 and S6) and other factors. Bivariate correlation analysis was performed on all data, and significant positive correlations were found between FC levels, and CRE (r = 0.924; p = 0.005), ESBL-resistant bacteria (r = 0.913, p = 0.010), TC (r = 0.921, p = 0.008), and bla\textsubscript{NDM-1} gene (r = 0.934, p = 0.009) concentrations in hospital wastewaters across the city (see Figure 1). FC levels also significantly correlated with int1 levels (r = 0.77, p = 0.04; see Figure S2), and with bla\textsubscript{CTX}, bla\textsubscript{OXA}, and bla\textsubscript{TEM} abundances among samples, but int1 did not significantly correlate with bla\textsubscript{NDM-1} (p = 0.25). Overall, data show a close association between fecal bacterial levels in hospital wastewaters, and levels of CRE and ESBL-resistance isolates and genes, which are released into the environment.

To better understand AR release patterns, wastewater data were segregated for each hospital per sampling season (see Figure 2, and Tables S5 and S6). Although not always statistically
significant, FC, CRE and ESBL-resistant bacterial abundances, and blaNDM-1 and int1 concentrations were always higher in hospital wastewaters in winter samples. Seasonal differences were greatest for blaNDM-1 abundances, which were three to nine orders of magnitude higher in the winter versus summer. Parallel hospital antibiotic use data was not available (functionally impossible to obtain in India), but seasonal differences almost certainly relate to differences in winter versus summer antibiotic use due to higher levels of infectious disease in the winter. Abundances of CRE also were higher in the winter, but differences between seasons were not as profound. This is possibly explained by individual phenotypes carrying multiple blaNDM-1 genes, which is common in extreme resistant phenotypes. Overall, results suggest greater exposures to blaNDM-1 exist in the winter from hospital wastes, which should be considered in public health AR risk assessments.

To further explain differences in blaNDM-1, other factors were examined. Inspection of AR data among hospitals hinted size may play a role in disproportionately higher releases in some hospitals. Therefore, ARG and ARB hospital wastewater data were sorted according to hospital size (i.e., small = 150-691 admissions, large = 1950-7270 admissions), and larger hospitals were found to release significantly higher concentrations of FC, CRE and blaNDM-1 on a mass-per-wastewater volume basis (see Figure 3). Paired t-test analysis shows FC (p = 0.046), CRE phenotypes (p= 0.009) and blaNDM-1 (p=0.009) (and also blaCTX, p=0.020; see SI) all were significantly higher in larger versus smaller hospitals. ESBL-resistant phenotypes (p = 0.065), blaOXA (p = 0.105) and blaTEM (p = 0.139) also showed increasing trends with hospital size.

A "hospital size" effect has been observed before in Sub-Saharan African hospitals, where larger hospitals contributed higher bacterial loads in their wastewaters compared with smaller hospitals, although no explanations were provided. It was initially suspected here overcrowding
in larger hospitals might explain this trend, with hospitals H1, H2, H5, H6 and H7 (all of which are large) reporting admittance rates three to four times their bed capacity (see Table S1).

However, many hospitals in India over admit. Therefore, differences between these five and the other hospitals are not definitive because, if a hospital does not report overcrowding, it not does mean overcrowding does not exist. Regardless, mean \(\text{bla}_{\text{NDM-1}}\) levels in wastewater from H1, H2, H5, H6 and H7 were significantly higher than the other seven hospitals (i.e., 11.29 ± 2.11 versus 8.87 ± 2.15 log (copy number/mL)), implying overcrowding may be important, although more work is needed to verify this speculation.

To place hospital wastewater AR data into a relative exposure context, five complimentary urban sewer drains in catchments without hospitals (to avoid bias) were sampled to quantity and contrast AR levels in “typical” Delhi community wastes (see Tables S7 and S8 for individual drain data). Significantly higher levels of TC, FC, ESBL-resistant, and CRE strains were present in hospital versus community wastes (\(p < 0.01\) based on paired t-tests) and, with one exception, all ARGs assayed were significantly higher in the hospital wastes (\(p < 0.02\)). Of particular note, however, is the huge difference in \(\text{bla}_{\text{NDM-1}}\) levels between hospital and community wastes in the winter; almost nine orders of magnitude greater. Given \(\text{bla}_{\text{NDM-1}}\) levels significantly correlate with 16S-rRNA gene levels in the hospital wastewaters (see Figure S3; ~2.3% of 16S-rRNA genes), data indicated particularly high levels of \(\text{bla}_{\text{NDM-1}}\) per cell (i.e., 16S-rRNA) are being released from hospitals, especially compared with \(\text{bla}_{\text{NDM-1}}\) in the community drains.

To place these ARG data into a medical context, extensive microbial culturing and isolation was performed to identify specific CRE and ESBL-resistant in the hospital wastes. In large hospital wastewaters, 953 CRE (Figure 4) and 748 ESBL-resistant (Figure S4) strains were identified, whereas 494 and 452 CRE and ESBL-resistant isolates, respectively, were identified.
in the small hospitals. *Klebsiella pneumoniae* (26%), *Pseudomonas putida* (19%) and *Klebsiella pneumonia subsp. pneumonia* (17%) were the most common CRE isolates in wastes from the large hospitals, whereas *Acinetobacter baumannii* (25%) and *Klebsiella pneumonia* (14%) were the predominant CRE phenotypes in the smaller hospitals (accession numbers are provided for isolates in Table S9). In terms of ESBL-resistant isolates, *E.coli* (26%) was predominant in the larger hospitals, whereas *Pseudomonas putida* (25%) was dominant in smaller hospitals. Further genetic characterisation of the isolates showed 55% of CRE isolates from large hospitals and 27% from small hospitals carried at least one \(\text{bla}_{\text{NDM-1}}\) gene; i.e., DNA from each isolate was amplified (using NDM-1-F and NDM-1-R) and amplicons sequenced, and relative \(\text{bla}_{\text{NDM-1}}\) prevalence was verified. The most common carriers of \(\text{bla}_{\text{NDM-1}}\) were *Klebsiella pneumoniae*, *E. coli*, *Acinetobacter baumannii* and *Pseudomonas putida*. Although these observations are not statistical, they indicate consequential carriage of \(\text{bla}_{\text{NDM-1}}\) in medically-relevant strains are being released to the wider environment in hospital wastewaters.

Of particular importance from a global health perspective is the high number of CRE isolates in Delhi hospital wastewaters on the WHO pathogen list in urgent need of new therapeutic options. The WHO recently published a list of “priority pathogens” in critical need of new antibiotics\(^{36}\), which include carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and ESBL-producing *Enterobacteriaceae*. Forty-one percent and 18% of our CRE isolates from small and large hospital wastewaters, respectively, are on the “critical” list, implying large numbers of functionally untreatable pathogens are being released to surface waters from New Delhi hospitals. Given the warm climate, such strains readily spread through the open drains, exposing millions of people to serious infectious disease.
Wider implications of these observations are not yet known. Nosocomial infections are a major cause of morbidity and mortality\textsuperscript{37}, and are a major challenge for public safety. Infections caused by gram-negative bacteria, such as has been detected and isolated here, are of particular concern since such microbes can be efficient in exchanging ARGs, including single genes (e.g., \textit{bla}\textsubscript{NDM-1}) that confer MDR to antibiotics. A recent report from United States highlighted that in ICUs these gram-negative bacteria are accountable for more than 70\% of the nosocomial infections (predominately ventilator-associated pneumonia and urinary tract infection), and similar statistics have been reported from other parts of the world\textsuperscript{38,39}. In 2002, the estimated number of nosocomial infections in U.S. was about 1.7 million and the number of deaths was estimated at \~99,000. Of these, 80\% of deaths were due to hospital acquired pneumonia (36\%), bloodstream infection (31\%) and urinary tract infections (13\%)\textsuperscript{37}. The increasing resistance of such gram-negative bacteria towards different antibiotics further worsens the situation, especially where the emergence of new AR is outcompeting development of new antibiotics\textsuperscript{40}.

The burden of AR continues to increase and is as a major threat to the public health, especially the treatment of infectious disease\textsuperscript{41}. Data herein show ARB and ARGs in hospital wastewaters in New Delhi pose a particular health risk because they include extremely MDR phenotypes, including “untreatable” pathogens. We suspect high levels are due to hospital overuse of critical antibiotics, overcrowding, and generally ineffective hospital wastewater treatment plants. As such, to reduce health risk, it is crucial more prudent hospital (and wider) antibiotic use be adopted, although installing and maintaining more effective hospital wastewater treatment systems also is needed. Treatment options that better target ARB and ARGs, including secondary and tertiary wastewater technologies, are needed as well as strategies for reducing HGT to environmental strains\textsuperscript{42}. Characterizing hospital wastes herein is an important starting
point for interventions, but use must be reduced and waste management improved in reduce AR
spread, especially in places like New Delhi without a fully developed urban wastewater
infrastructure.

Supporting Information

Additional information has been provided online, including: a list of hospitals where wastewater
samples were collected (Table S1); water quality conditions for samples collected from hospitals
and community drains (Table S2); primers for target genes in PCR and qPCR (Table S3); design
discharge levels from hospital wastewater treatment plants and community drains (Table S4);
detailed abundance data on TC, FC, and ESBL-resistant and CRE bacteria in wastewater
samples from the hospitals in the summer and winter (Table S5); abundances of 16S-rDNA
genes, blaNDM-1, blaCTX, blaOXA, blaTEM and intI in hospital wastewater samples in the summer
and winter (Table S6); abundances of TC, FC, and ESBL-resistant and CRE bacteria in sewer
drain samples in the summer and winter (Table S7); abundances of blaNDM-1, blaCTX, blaOXA and
blaTEM in sewer drain samples in the summer and winter (Table S8); and accession numbers for
CRE isolates from this study (Table S9). Also included are figures displaying the following:
relationships between ESBL-resistant bacteria and FC levels, and the relative abundance of
blaCTX, blaOXA and blaTEM, in hospital wastewaters (Figure S1); relationships between intI gene
abundances, and FC and blaNDM-1 levels in hospital wastewaters (Figure S2); relationships
between blaNDM-1 and 16S-rRNA gene levels in hospital wastewaters (Figure S3); and
proportional abundances of ESBL-resistant isolates as a function of hospital size in the hospital
wastewaters (Figure S4).
Authors contribution: SZA planned the experiment, SZA and ML collected the samples, ML analysed the samples, ML, SZA and DWG analysed data and prepared the manuscript.

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Figure Legends

Figure 1 Relationships between carbapenem-resistant Enterobacteriaceae (CRE), $bla_{NDM-1}$ abundance, ESBL-resistant bacteria (ESBL), and total coliform bacteria (TC), and fecal coliform bacterial levels in hospital wastewater outfalls from across New Delhi.

Figure 2 Concentration of FC, CRE, $bla_{NDM-1}$, $int1$ in wastewaters from each New Delhi hospital. Data normalized to patient number. Error bars refer to standard errors.

Figure 3 Abundance of faecal coliforms, CRE, $bla_{NDM-1}$ and $int1$ in wastewaters from large (n=6) and small (n=6) hospitals in New Delhi in winter samples. *Statistically significant difference (Paired t-test; CRE and $bla_{NDM-1}$, p = 0.009; Faecal Coliforms, p = 0.046). Boxes represent the 25 to 75% percentile range and bars represent the standard error.

Figure 4 Proportional abundance of CRE bacteria as a function of genus and hospital sizes in hospital wastewaters across New Delhi. Data reported per wastewater volume basis. Small hospital CRE isolates, n = 494; Large hospital CRE isolates, n = 953.
Hospital wastes seed carbapenem-resistance pathogens to Indian urban water systems
Figure 1
Figure 2
Figure 3
Figure 4