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Carbapenem resistance exposures via wastewaters across New Delhi

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ABSTRACT

Antimicrobial resistance (AMR) is a major global concern, especially in India where the burden of infectious diseases is high and health care spending is low. Here we quantified total coliform, faecal coliforms (FC), carbapenem-resistant enteric bacteria (CRE), bla_{NDM-1}, and three integron genes in samples collected from wastewater effluent of 12 hospitals, 12 sewage treatment plants (STPs), 20 sewer drains, and five locations along the Yamuna River in New Delhi over two seasons. Significant correlations were found between FC levels, CRE ($r = 0.903$, $p = 0.004$, $n = 49$) and bla_{NDM-1} ($r = 0.787$, $p = 0.003$, $n = 49$) concentrations across all samples. Concentrations of coliforms, CRE, bla_{NDM-1}, *int1*, and *int3* were highest in hospital effluents compared to other locations in both seasons. Although absolute concentration data indicate greater abundances of CRE and bla_{NDM-1} in the winter, normalised data indicates greater carriage of bla_{NDM-1} per cell in summer samples. In general, observed CRE levels were highest in surface water downstream of areas with higher population densities. Among CRE isolates ($n = 4077$), 82%, 75%, 71% and 43% of the strains from hospitals, sewer drains, river samples, and STPs, respectively, contained bla_{NDM-1}, implying STPs have relatively fewer bla_{NDM-1} positive CRE in their effluents. The most common CRE isolates in the drains were *Pseudomonas putida* (39%) followed by *Acinetobacter baumanni* (20%) and *Pseudomonas montelli* (19%). The present scenario in New Delhi highlights the urgent need for increased coverage of appropriate waste treatment facilities across the city to reduce CRE exposures from polluted surface waters.

1. Introduction

Increasing antimicrobial resistance (AMR) is a global concern in human and veterinary healthcare systems. AMR problems are greatest in emerging countries like India where infectious disease levels are high and per capita healthcare spending is low (Public Health Foundation of India, 2011). Although AMR is intrinsic in nature (D'Costa et al., 2011), continued use of antibiotics, pollution, and other factors have selected and mobilised AMR genes (ARGs) across the microbiome, including the acquisition of antibiotic resistance genes (ARGs) in strains of health importance. The mobilisation is largely fuelled by promiscuous mobile genetic elements (MGEs), including plasmids, transposons, and integrons (Mazel, 2006), which mediate AMR spread via horizontal gene transfer (HGT), creating resistant and multi-resistant phenotypes in pathogenic and non-pathogenic strains. Until fairly recently, AMR evolution and transmission was assumed to be a consequence of medical and veterinary antimicrobial use (Jin et al., 2017). However, it is now apparent that consequential AMR dissemination and exposure also occur away from places of antibiotic use through environmental pathways (Arcilla et al., 2016), such as via wastewater releases, and associated contaminated water and food (Christgen et al., 2015).

One place where AMR spread in the environment is most evident in New Delhi, which is where New Delhi metallo-β-lactamase protein (i.e., NDM-1) was first detected in 2008 in a carbapenem-resistant *Klebsiella pneumonia* isolate from a hospital patient (Yong et al., 2009). NDM-1 confers multi-resistance to many bacteria, including therapeutically critical carbapenems (Papp-Wallace et al., 2011). Unfortunately, bla_{NDM-1} (gene that codes for NDM-1) was detected soon thereafter in New Delhi surface waters and seeps in 2010, presumably associated with faecal releases (Walsh et al., 2014), and bla_{NDM-1} and variants are now found in patients and the environment in over 100 countries (Kumarasamy et al., 2010; Nordmann et al., 2011a, 2011c; Wilson and Chen, 2012). Phenotypes include strains functionally resistant to all antibiotics, including colistin and tigecycline (Kumarasamy et al., 2010; Nordmann et al., 2011b). Clinical evidence suggests antibiotics select for bla_{NDM-1} in gut strains and the gene subsequently enters surface waters, probably via human wastes (Hawkey and Jones, 2009). The bla_{NDM-1} gene has spread globally via international travel in exposed

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individuals (Hawkey and Jones, 2009; Petersen et al., 2015).

Although the above global pathway is probable, the main originating cause of bla_{NDM-1} within the New Delhi urban environment is unclear, especially the relative role of community, hospital, and sewage treatment plant (STP) effluents as sources of bla_{NDM-1} and carbapenem-resistant enteric bacteria (CRE) across the city. Therefore, we quantified levels of bla_{NDM-1}, three integron-related genes (*int1*, *int2*, and *int3*), 16S-rRNA genes, total coliforms (TC), faecal coliforms (FC), and CRE isolates in New Delhi surface waters at 49 locations in winter and summer samples.

Integrons are defined here as mobile gene cassettes composed of a gene-encoding integrase (e.g., *int*1), a recognition and recombination of cassette (att I) and a promoter (Pc) for expression of the cassettes (Hall and Collis, 1995). Based on previous data, class 1 markers tend to be most associated with waste releases followed by class 3 and class 2 markers (Rapa and Labbate, 2013). *int1* and *int3* are often found in freshwater and soil proteobacteria, whereas *int2* is a part of marine γ-proteobacteria (Deng et al., 2015; Li et al., 2013). Quantifying the abundance of integron gene cassettes is useful in potentially understanding bla_{NDM-1} dispersal because they often are associated with faecal releases to the environment (Andersson and Levin, 1999; Courvalin, 1994; Gillings et al., 2015; Kruse and Srum, 1994; Leverstein-Van Hall et al., 2002). These data were used to quantify season differences in environmental CRE and bla_{NDM-1} exposure, the relative contribution of community, hospital and STP wastewater sources to proximal levels, and dominant CRE species found in sewer drains and the Yamuna River that impacts residents via environmental exposures.

This study is globally relevant because India is the largest consumer of antibiotics for personal use in the world and β-lactams are among the most commonly used antibiotics in India (Van Boeckel et al., 2014). We suspect antibiotic misuse in places like India partially explains the early evolution of CRE strains, including bla_{NDM-1} positive isolates. Therefore, although New Delhi presents an extreme case, it is a template for understanding AMR spread in any large city without adequate wastewater management; a common scenario in the developing and emerging world.

2. Materials and methods

2.1. Study area and sampling program

Sampling across the New Delhi wastewater network included hospital effluents, open and sub-surface sewer drains, STPs and final receiving waters. The network comprised 20 drain sites, 12 hospital waste outfalls, effluents from 12 STPs, and five sites along the Yamuna River, which is the ultimate receptacle for wastewaters from the city (see Supporting Information (SI) Fig. S1 and Tables S1–S3 for locations and details). Due to the myriad of wastewater sources to the network, it was not possible to exactly quantify contributing population numbers (i.e., as cohorts) or hospital versus community antibiotic use in an exact manner. Therefore, comparisons among community, hospital, and STP sources were based on detected bla_{NDM-1}, other genes and bacterial numbers per wastewater catchment area. This allowed a block experimental design that allowed two-sample comparisons of genes and bacteria per catchments or locations with different contributors.

Water column samples were collected in January/February ("Winter") and May/June ("Summer") 2014 from all locations. The following were quantified in samples (at least in triplicate): TC, FC, and CRE colony forming units (CFUs), and temperature (T), pH, total dissolved solids (TDS), and specific conductivity. Water samples were subsampled in triplicate for qPCR analyses of bla_{NDM-1}, integron 1, 2 and 3 gene cassette markers (*int1*, *int2*, and *int3*), and 16S rRNA genes.

2.2. Initial sample collection and processing

All the samples were collected in sterile 500-mL containers and returned to laboratory transported on ice for subsequent microbial and molecular biological analysis. All microbial plating and culturing work was performed within 24 h of sampling, whereas sub-samples for molecular work were frozen at −20 °C for subsequent DNA extraction and qPCR. At the time of sampling, wastewater temperature, pH, TDS, and specific conductivity were measured to describe the water conditions under which samples for the microbiological and molecular biological analyses were collected.

2.3. Microbial culturing and plating

Samples for TC, CRE, and FC were serially diluted in sterile phosphate buffer solution (PBS) and plated in triplicate (per dilution) on Rapid HiColiform Agar (Himedia, India), HiCrome KPC Agar Base (Himedia, India) at 37 °C for 24 h, and M-FC Agar Base (Himedia, India) at 45 °C for 24 h, respectively. CFUs were estimated according to manufacturer's instructions. Resistant colonies were selected from the KPC plates and re-streaked to purity to allow 16S sequencing and phenotyping. Isolate identities were verified using strain-specific Biochemical Test Kits (Himedia, India) and included more than just KPC strains. Therefore, identified carbapenem-resistant isolates are referred to as CRE herein.

2.4. DNA extraction and qPCR assays

DNA from frozen water and wastewater samples, and pure culture isolates were extracted using the Fast Soil DNA extraction kit and a Ribolyzer according to manufacturer's instructions. DNA from pure cultures was amplified using bacterial 338F and 1046R primers (Huber et al., 2007; Yu et al., 2005); preheating at 95 °C for 5 min, 39 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 45 s and final extension at 72 °C for 7 min. Agarose gels were run to examine the primary PCR products and the 16S-rRNA was sequenced. Extracted DNA from the field samples was stored at −20 °C for subsequent qPCR analysis. Specific genes reported here include bla_{NDM-1}, *int1*, *int2* and int3, and 16S-rRNA genes were also quantified to estimate the total eubacterial population size. The probes/primers used in qPCR are provided in Table S4 (SI).

All genes were quantified in triplicate using the BioRad CFX-96 system (BioRad, USA). All reactions were performed with serially-diluted DNA standards and DNA-free negative controls. Correlation coefficients for the calibration curves were > 0.99 and all the log gene abundance values were in the linear range of detection.

2.5. Isolate identification and characterization

To identify and characterize the CRE isolates, pure cultures were developed, DNA extracted, and 16S-rRNA (Pace, 1997) were amplified using bacterial 338F and 1046R primers. Purified PCR products were sequenced using Sanger Shotgun sequencing. Sequences were compared with the GenBank database (Benson et al., 2013) using Standard Nucleotide BLAST tool, and strains were identified based on percentage similarities with strains within the database. Biochemical test kits were used to confirm isolates and all isolates were screened for carriage of bla_{NDM-1} using PCR.

2.6. Statistical analysis and data visualization

All the data analysis was done using Excel 2007 (Microsoft Corporation, USA) and SPSS Version 19.0 (Chicago, IL). Point data (e.g., CRE concentrations) were analyzed using bivariate correlation employing the Spearman's non-parametric methods on log-transformed data because normality could not be assumed for all datasets. The

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