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Increased Waterborne bla_{NDM-1} Resistance Gene Abundances Associated with Seasonal Human Pilgrimages to the Upper Ganges River

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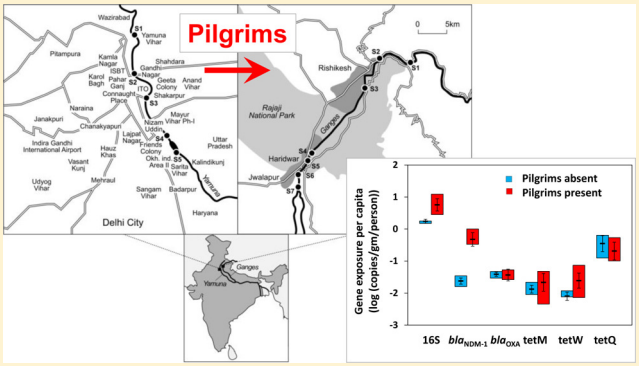
Supporting Information

ABSTRACT: Antibiotic resistance (AR) is often rooted in inappropriate antibiotic use, but poor water quality and inadequate sanitation exacerbate the problem, especially in emerging countries. An example is increasing multi-AR due to mobile carbapenemases, such as NDM-1 protein (coded by bla_{NDM-1} genes), which can produce extreme drug-resistant phenotypes. In 2010, NDM-1 positive isolates and bla_{NDM-1} genes were detected in surface waters across Delhi and have since been detected across the urban world. However, little is known about bla_{NDM-1} levels in more pristine locations, such as the headwaters of the Upper Ganges River. This area is of particular interest because it receives massive numbers of visitors during seasonal pilgrimages in May/June, including visitors from urban India. Here we quantified bla_{NDM-1} abundances, other AR genes (ARG), and coliform bacteria in sediments and water column samples from seven sites in the Rishikesh-Haridwar region of the Upper Ganges and five sites on the Yamuna River in Delhi to contrast bla_{NDM-1} levels and water quality conditions between season and region. Water quality in the Yamuna was very poor (e.g., anoxia at all sites), and bla_{NDM-1} abundances were high across sites in water (5.4 ± 0.4 log(bla_{NDM-1}·mL^{-1}); 95% confidence interval) and sediment (6.3 ± 0.7 log(bla_{NDM-1}·mg^{-1})) samples from both seasons. In contrast, water column bla_{NDM-1} abundances were very low across all sites in the Upper Ganges in February (2.1 ± 0.6 log(bla_{NDM-1}·mL^{-1})), and water quality was good (e.g., near saturation oxygen). However, per capita bla_{NDM-1} levels were 20 times greater in June in the Ganges water column relative to February, and bla_{NDM-1} levels significantly correlated with fecal coliform levels ($r = 0.61$; $p = 0.007$). Given that waste management infrastructure is limited in Rishikesh-Haridwar, data imply bla_{NDM-1} levels are higher in visitor's wastes than local residents, which results in seasonally higher bla_{NDM-1} levels in the river. Pilgrimage areas without adequate waste treatment are possible "hot spots" for AR transmission, and waste treatment must be improved to reduce broader AR dissemination via exposed returning visitors.



INTRODUCTION

Great concern exists over the transmission of mobile carbapenemases to pathogens, which are enzymes that confer antibiotic resistance (AR) to carbapenems and other β-lactam antibiotics and multiresistance to other antibiotics.[1] Of particular concern are plasmid-borne genes, such as bla_{NDM-1} that code for the NDM-1 metallo-β-lactamases, which readily migrate among bacteria and can result in extreme drug-resistant phenotypes.[2,3] Until recently, resistant strains that carry bla_{NDM-1} were found only in clinical settings,[4] but surface water sampling in Delhi in 2010 showed that resistant organisms and bla_{NDM-1} genes also were present in surface waters.[5] As such, water-related bla_{NDM-1} exposure is possible via contaminated urban water. Since these early discoveries, bla_{NDM-1} genes have now been found elsewhere in the world.[6] However, major pathways for widespread bla_{NDM-1} dissemination are not apparent, although international travel and tourism have been implicated.[7,8] Unfortunately, although rapid dissemination of bla_{NDM-1} genes is happening, data are still lacking for epidemiological assessments, and it is critical to identify scenarios for quantifying the spread of bla_{NDM-1} from environmental and other sources to pathogens of clinical importance.

One scenario where quantitative transmission assessments are possible is associated with the seasonal mass migration of pilgrims from large cities in India to religious shrines in pristine areas, such as sacred sites along the Upper Ganges River. Cities

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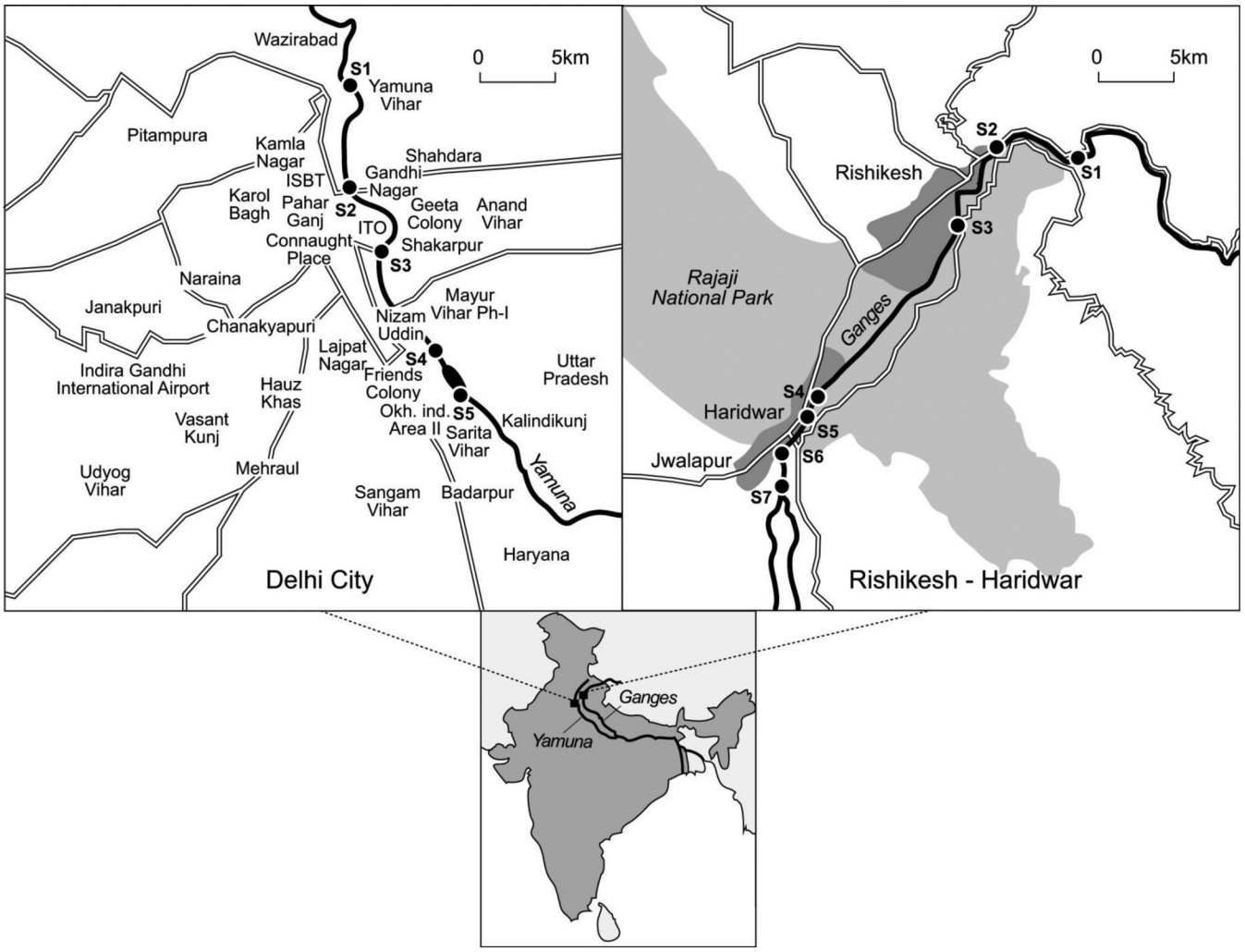

Figure 1. Sampling sites on the Upper Ganges River in the Rishikesh-Haridwar region and the Yamuna River in Delhi.

such as Rishikesh (pop. 78,805; 2011 census) and Haridwar (pop. 220,767) in the foothills of the Himalayas receive up to 500,000 additional visitors in May and June, providing a large influx of nonresidents who may carry NDM-1 positive strains in their guts. Unfortunately, waste treatment is limited in such areas (e.g., Rishikesh-Haridwar treatment plant capacity is about pop. 80,000); therefore large quantities of inadequately treated wastes are released into the Upper Ganges, especially during the pilgrimage season. The potential for increased $bla_{\text{NDM-1}}$ exposure is problematic because pilgrims generally presume water quality in the Upper Ganges is good, not considering how their mass visits impact local water quality. Therefore, by comparing fecal coliform and $bla_{\text{NDM-1}}$ abundances in waters and sediments before and during the pilgrimage season, one can determine possible exposure risks to visitors and residents during pilgrimages, which can be used to guide new waste management approaches. This is critical from a health perspective because water consumption and bathing are key elements of pilgrimages to the region.

Here we used seasonal visitor and resident population data in conjunction with water quality, quantitative molecular microbiology on AR gene (ARG) abundances (including $bla_{\text{NDM-1}}$), and fecal microbial indicators to assess human impacts to ARG before and during the pilgrimage season. Specifically, we compared $bla_{\text{NDM-1}}$, other ARG, and total and fecal coliform levels in sediment and water samples between seasons at selected sites on the Upper Ganges River near Rishikesh-Haridwar and on the Yamuna River in Delhi (Figure 1). The goal was to determine whether $bla_{\text{NDM-1}}$ and other ARG levels differ between urban Delhi and pristine Rishikesh-Haridwar and to assess the potential for broader AR transmission associated with pilgrimages.

METHODS

Study Area. The Ganges River is 2525 km long, and its watershed covers more than one million km^2 (about 25% of India's total geographical area), spanning India, Nepal, Bangladesh, and China. The Upper Ganges River, which is the focus of this study, originates from glaciers at Gaumukh and enters the northern Indian plain at Rishikesh, a small town with many religious shrines and ashrams near the river. Below Rishikesh, the river flows ~30 km south to the city of Haridwar (which translates to "Gateway to Lord Vishnu"), the first larger city on the river's path across the Upper Ganges basin. Within this region, seven shoreline sites were sampled, including three nearer to Rishikesh and four nearer to Haridwar. To contrast conditions with these sites, five sites were chosen and sampled along the Yamuna River, which flows through Delhi. The urban sites were chosen to span the city and avoid clear pollutant outfalls, which are numerous on the river. Specific sampling

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Table 1. Seasonal Water Quality Conditions in the Upper Ganges River near Rishikesh-Haridwar and Yamuna River in Delhi

	DO (mg/L)		TDS (mg/L)		temp (°C)		pH	
	Feb	Jun	Feb	Jun	Feb	Jun	Feb	Jun
Rishikesh	8.9 (0.06)[a]	9.4 (0.20)	68.3 (4.3)	71.7 (5.4)	14.0 (0.13)	19.9 (0.47)	6.8 (0.60)	7.1 (0.20)
Haridwar	9.7 (0.30)	9.0 (0.30)	81.3 (3.0)	107 (21.6)	16.2 (0.58)	22.6 (0.58)	7.5 (0.60)	7.6 (0.50)
Delhi	2.8 (0.56)	2.2 (0.58)	355 (64.8)	504 (102)	14.9 (0.46)	32.1 (1.04)	8.2 (0.21)	8.1 (0.14)

[a]95% confidence intervals.

sites on the Upper Ganges and Yamuna Rivers are shown on Figure 1.

Sample Collection and Initial Processing. Sediment and water column samples were collected from both rivers in February and June 2012. All field samples were collected in triplicate at each site in sterile 500-mL containers (VWR, U.K.) and returned to the laboratory on ice in coolers, which was similar to previous field studies.[9,10] Upon return to the lab, aliquots were aseptically transferred into microcentrifuge tubes and frozen at −20 °C for subsequent molecular biological analysis. The remaining samples were used for microbial plating and culturing work, which was performed within 24 h of sampling. Dissolved oxygen (DO), pH, total dissolved solids (TDS), and temperature were measured in situ using hand-held field probes (Waters, Germany).

Microbial Culturing of Fecal and Total Coliform Bacteria. Fecal and total coliform plate counts were performed on sediment and water column samples from all sites. Culturable organisms were extracted from sediment samples using a sterile buffer solution prepared by dissolving 0.2% (w/v) sodium pyrophosphate in ultrapure water. The extraction was performed by combining wet sediment sample and buffer solution at a 1:1 ratio (v/v) in sterile 50-mL Falcon tube and providing agitation for 4 h at 120 rpm and 4 °C. This mixture was then serially diluted using sterile phosphate buffer solution (PBS) and plated in triplicate (per dilution) on Rapid Hi-chrome coliform agar (Himedia, India) at 37 °C then 44 °C for 24 and 48 h, respectively. Raw water column samples were diluted directly in sterile PBS and plated identically to sediment samples onto the same agar. The fecal and total coliform colony forming units (CFU) were estimated according to manufacturer's instructions.

DNA Extraction and qPCR Detection of ARG. DNA was extracted within 24 h of sample collection using the Fast Soil DNA extraction kit (MP Biomedicals, USA) and a Ribolyzer (MP Biomedicals, USA), according to manufacturer's instructions. Specifically, extracted DNA samples were stored at −20 °C in India and then returned frozen to the United Kingdom for subsequent qPCR analysis. Specific genes targeted included $bla_{\text{NDM-1}}$;[4,11] bla_{OXA} (a widespread class D β-lactamase gene);[12,13] and three common tetracycline resistance genes, *tet*(Q), *tet*(W), and *tet*(M)[15,16] found in human and other mammalian guts.[17] Along with these ARGs, the abundance of 16S-rRNA genes[18,19] also was quantified to estimate total bacterial population size for normalization of ARG abundances relative to total bacterial community size.

All genes were quantified in triplicate using qPCR on a BioRad CFX C1000 System (BioRad, Hercules, CA USA) and established probes and primers for targeted ARGs (see Supporting Information, Table S1). Each 10-μL reaction mixture contained 3 μL of template DNA, 1 μL of primers (10 pmol/μL), 1 μL of nuclease free water, and 5 μL of qPCR reagent (SsoFast EvaGreen Supermix, BioRad, USA). The programs were as follows: bacterial 16S-rRNA and tetracycline resistance genes *tet*(M), *tet*(Q), and *tet*(W) had initial enzyme activation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 45 s; bla_{OXA} had initial enzyme activation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 5 s, and annealing and extension at 55 °C for 10 s, and $bla_{\text{NDM-1}}$ had initial enzyme inactivation at 95 °C for 5 min, 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min.

All reactions were run in parallel with serially diluted DNA standards of known quantity and DNA-free negative controls. PCR efficiencies (always ∼87−105%) were determined by comparing signals from serial dilutions of samples with high abundance of DNA with plasmid controls. Calibration curves yielded $R^2 > 0.99$, and log-transformed gene-abundance values always were in the linear range. The presence of inhibitory substances in the extracted DNA samples was checked by spiking known amounts of template DNA in UV-treated samples and comparing differences in concentration threshold (CT) values among the samples and neat positive-controls (one cycle difference between samples and controls was targeted). Based on pretesting, the extracted DNA were diluted (1:100) with molecular-grade, nuclease-free water before being analyzed using qPCR to minimize inhibitory effects of extraneous matter in the samples. To verify that the $bla_{\text{NDM-1}}$ probes were actually targeting carbapenemase-associated sequences in water samples, selected PCR amplicons were sequenced, and all DNA sequences were found consistent with the intended targets.

Data Analysis. All data analyses were conducted using SPSS (Chicago, IL; v. 17.0) and Excel 2010 (Microsoft Office 2010, Microsoft Corp., USA). All data were log-transformed to improve sample normality before analysis. Statistical significance was always defined by 95% confidence intervals ($p < 0.05$).

RESULTS

River Water Quality in the Upper Ganges and Yamuna Rivers between Seasons. Water column and river sediment samples were collected in February and June 2012 at seven locations on the Upper Ganges River near Rishikesh-Haridwar and at five locations on the Yamuna River near Delhi (see Figure 1). Rishikesh and Haridwar are located in the Himalayan foothills where residential human population densities are low and there is minimal agricultural activity. The Upper Ganges is primarily fed by glacial and snowmelt from the mountains; however, it also receives increased rainfall inputs during the monsoon season (July to September). In 2012, flow rates in June were about double those in February, which is typical of the Upper Ganges at that time of year.

Given the pristine upland watershed, water quality conditions in the Upper Ganges River are generally good, which was confirmed by our monitoring data (Table 1). High ambient dissolved oxygen (DO; 8.9−9.6 mg/L) and low total dissolved

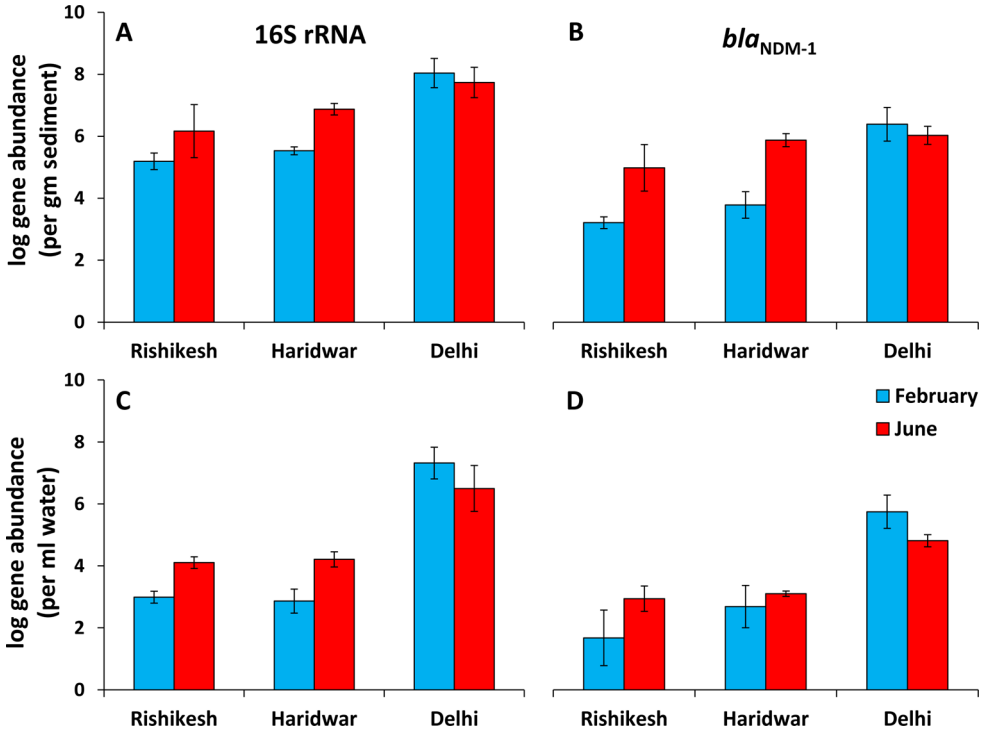

Figure 2. log(16S rRNA bacterial gene) and log(bla_{NDM-1}) abundances in sediment and water column samples collected in February (blue) and June (red) near Rishikesh, Haridwar and Delhi on the Upper Ganges and Yamuna Rivers. (A) 16S and (B) bla_{NDM-1} levels are provided per gram dry weight of sediment collected, whereas (C) 16S and (D) bla_{NDM-1} represent levels per mL of water column sample. Error bars present standard errors.

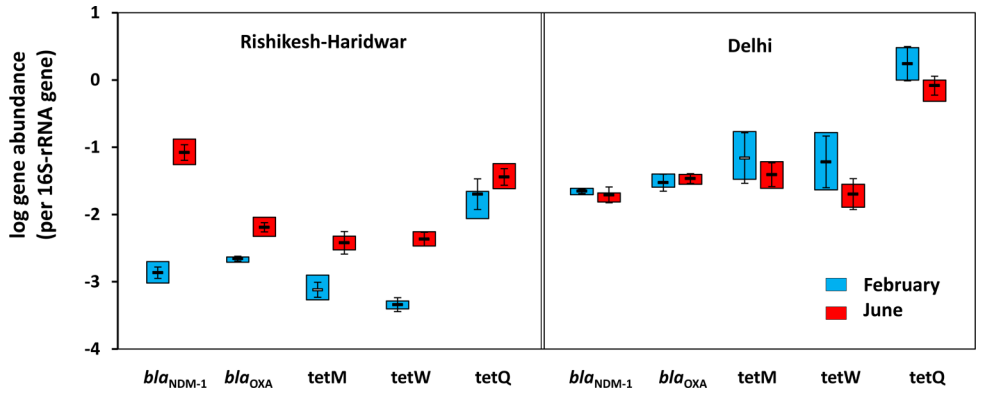

Figure 3. Relative abundances of bla_{NDM-1}, bla_{OXA}, *tet*(M), *tet*(W), and *tet*(Q) resistance genes normalized to ambient 16S rRNA bacterial gene levels in sediment samples collected in February and June 2012 from the Rishikesh-Haridwar area of the Upper Ganges River and in Delhi on the Yamuna River. Boxes represent 25−75% quartile ranges, and error bars indicate the standard errors.

solids levels (TDS; 68−107 mg/L) suggest the river is well oxygenated and has minimal extraneous organic and inorganic matter. TDS levels were slightly higher in June versus February (107 vs 81.3 mg/L at Haridwar sites; Table 1), which was probably due to greater human waste inputs during the pilgrimage season. As background, pilgrims usually visit the region for a few weeks to a month, staying in hotels, dharamshala (spiritual dwellings), guest houses, temples, and private accommodations, which overall taxes local waste management facilities.

In contrast, water quality in the Yamuna River in Delhi was very poor both in February and June. DO levels approached zero, and TDS levels were five times greater than levels seen in the Upper Ganges (i.e., 504 mg/L vs ≤107 mg/L; Table 1). The Yamuna receives massive inputs of agricultural, industrial, and domestic pollutants[20] with some reaches along the river having a septic odor. This suggests anoxic conditions prevail near the water surface, which is unusual for such a large river that typically would have greater dilution, better mixing, and more reaeration during normal flow conditions.

Resistance Genes in River Waters and Sediments. Replicate water column and sediment samples were collected from both rivers, and bla_{NDM-1}, bla_{OXA}, *tet*(M), *tet*(Q), *tet*(W), and 16S-rRNA gene abundances were quantified using qPCR. All ARG and 16S gene abundances were 2−3 orders of magnitude higher in sediments than the water column at all Upper Ganges River sites, assuming specific gravity of the sediment mass is roughly 2.5 times that of water (see Supporting Information, Tables S2 and S3). However, ARG levels were significantly higher in both water column and sediment samples in June relative to February levels (Wilcoxon Two-sample nonparametric test (WT); $p < 0.01$), which is

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consistent with increased human waste releases when the seasonal visitors are present. However, the largest seasonal increase in ARG was seen in sediment-associated bla_{NDM-1} levels (see Figure 2), which parallels bacterial 16S-rRNA gene levels at the same sites.

In contrast, water column and sediment ARG levels were always elevated in the Yamuna River (Figure 2 and Tables S1 and S2), with significantly higher ARG levels in the water column relative to the Ganges (e.g., bla_{NDM-1}; WT, $p < 0.01$). High water-column ARG levels are consistent with many waste outfalls into the river, which has been seen elsewhere in the world[9] and also known for the Yamuna River. Importantly, although ARG concentrations tended to be slightly lower in February than June in the Yamuna, statistically significant differences in ARG levels between seasons were not seen (WT, $p > 0.05$), which is different than seen in the Upper Ganges River near Rishikesh-Haridwar.

To place these data into context, sediment ARG levels were normalized to 16S-rRNA bacteria gene levels and combined for all Rishikesh-Haridwar and Delhi sites (Figure 3). Normalizing to 16S-rRNA gene levels describes the level of local enrichment of ARG relative to the total bacterial population. In the Upper Ganges, all measured ARG were significantly more enriched in June relative to February (WT; $p < 0.05$). However, the extent of enrichment was greatest for bla_{NDM-1} relative to the other ARGs, suggesting there is a disproportionately larger seasonal input of "new" bla_{NDM-1} to Upper Ganges sediments in June when visitors are present. In contrast, no significant seasonal difference in ARG enrichment was seen in Yamuna River sediments (WT > 0.05). In fact, normalized ARG levels were lower in June than February, probably resulting from higher volumetric flow rates in the river in June (about double) and greater dilution. ARG levels were high year-round in the Yamuna River, which reflects large and chronic waste inputs into the river.[20] In contrast, elevated normalized ARG levels were only high in the Upper Ganges when visitors were present, especially bla_{NDM-1}.

Such differences might be explained by the larger human populations present in June, but when one normalizes ARG abundances to human population size in the region (including visitors), significantly greater bla_{NDM-1} and 16S rRNA gene abundances per capita were apparent in June in the Upper Ganges River (see Figure 4; WT; $p < 0.01$). Interestingly, the other four ARG tested did not significantly vary seasonally on a per capita basis (WT; $p > 0.05$), implying these "older" ARG are likely endemic in the gut and wastes of visitors and residents alike.[17] However, bla_{NDM-1} is a more recently detected ARG in human gut flora,[3,4] and Figure 4 shows it is over 20 times greater in the river (per capita) in June relative to February. This suggests that visitor wastes, which are more apparent in June, have higher intrinsic bla_{NDM-1} abundances than local residents, i.e., elevated bla_{NDM-1} levels in the river in June are primarily associated with waste inputs from the visitors.

Relationships between Fecal and Total Coliforms and bla_{NDM-1} Abundances. To confirm relationships between high bla_{NDM-1} levels and fecal releases in the river, total coliform (TC) and fecal coliform (FC) bacteria were cultured from Upper Ganges and Yamuna River water column and sediment samples. bla_{NDM-1} levels significantly correlated with FC levels among all sites ($r = 0.61$, $p = 0.007$; Figure 5), indicating bla_{NDM-1} in the river water is likely associated with fecal matter and waste releases. Interestingly, although a positive trend also existed between FC and bla_{NDM-1} in sediments at the sites, the correlation was not statistically significant ($r = 0.24$, $p = 0.211$). This implies sediment FC and bla_{NDM-1} are still associated, but relationships weaken because fecal culturable organisms, and specific genes can become less connected within exposed sediments.[9] Further, a positive trend also existed between TC and bla_{NDM-1} in the water column among sites (Figure 5; $r = 0.34$, $p = 0.167$). This is not surprising because environmental TC can have sources other than fecal matter.

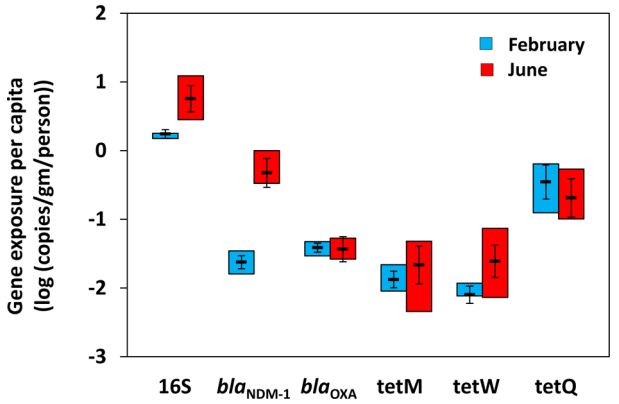

Figure 4. Sediment gene abundances of 16S rRNA gene, bla_{NDM-1}, bla_{OXA}, *tet*(M), *tet*(W), and *tet*(Q) normalized to estimated human populations in the Rishikesh-Haridwar area in February (local residents only) and in June (visitors plus local residents). Human populations are assumed to be 320,000 and 820,000, respectively. Only 16S rRNA and bla_{NDM-1} per capita levels significantly differ between seasons, indicating visitor wastes contain more bla_{NDM-1} than local residents. Boxes present the 25−75% quartile range, and error bars show standard errors.

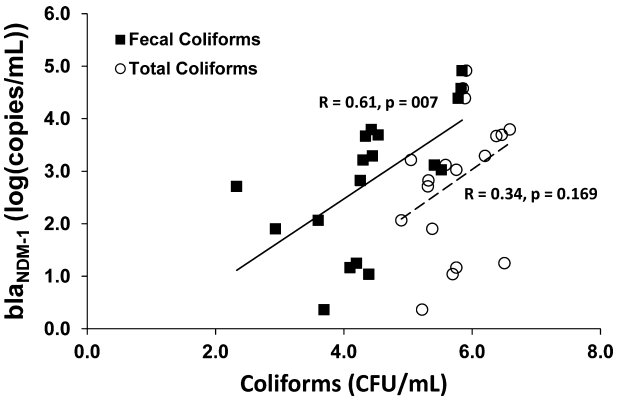

Figure 5. Water column bla_{NDM-1} abundances and fecal (FC) and total (TC) coliform bacterial levels for all samples collected in both the Upper Ganges and Yamuna Rivers during February and June sampling events.

DISCUSSION

Antibiotic resistance is ancient,[21] but AR levels are increasing worldwide due to increased use of antibiotics and many other factors.[10,22−25] However, there has been considerable debate about the dominant mechanisms by which AR is transmitted around the world.[26] Recent evidence has shown that dissemination of AR can be rapid.[27] For example, bla_{NDM-1} gene and its protein was first seen in patients in India about six years ago[4] and is now detected in patients around the world, including many new variants.[28] Original discovery of strains carrying bla_{NDM-1} was in hospital settings, but it has since been

detected in surface waters,[7] implying this plasmid-borne gene is migrating outside of hospital environments, including waters, soils, and sediments.[29]

Here, we compare bla_{NDM-1} and other ARG levels in human waste-impacted waters from a pristine region of India (Rishikesh-Haridwar) and a location where bla_{NDM-1} has been broadly detected in surface waters (Delhi), using PCR primers designed on the basis of Indian fecal samples. The data suggest that permanent residents of Rishikesh-Haridwar carry lower levels of bla_{NDM-1} genes in their gut (and wastes) relative to seasonal visitors to the region (see Figure 4). In fact, the "average" visitor appears to carry at least >20 times more bla_{NDM-1} genes than Rishikesh-Haridwar residents in 2012, and increased waterborne bla_{NDM-1} exposure occurs during the pilgrimage season, both to pilgrims and local residents. As such, pilgrimage areas may act as "hot spots" for the broader transmission of bla_{NDM-1} and other ARG, especially considering bathing and water consumption occur in Ganges waters and exposed visitors return home after their visit to the region.

One might argue that our bla_{NDM-1} data do not solely represent human waste releases to the river and could be seasonally elevated for other reasons. However, other ARG data suggest otherwise (Figure 3 and 4, and Supplementary Tables S2 and S3). Sediment bla_{OXA}, *tet*(W), and *tet*(M) levels in the Upper Ganges are roughly the same in February and June, which is consistent with the probable presence of such genes in the gut and wastes from both visitors and residents. Further, one might contend waste releases to the river may be impacted by nonhuman animal wastes, such as cattle, which also contain elevated bla_{OXA}, *tet*(W), *tet*(M), and *tet*(Q). However, *tet*(Q) tends to be less associated with human wastewater,[30] and there is significant decrease in per capita *tet*(Q) levels in June, implying *tet*(Q) in the river is not as strongly linked with seasonal human populations. Finally, FC and bla_{NDM-1} significantly correlate in the river water column (Figure 5), including periods when per capita bla_{NDM-1} levels were elevated; therefore, higher bla_{NDM-1} levels in June are likely associated with greater quantities of visitor fecal matter in the Upper Ganges.

These results have two major implications. First, bla_{NDM-1} genes do not appear to be equally present across the Indian population. Our data suggest results from previous studies,[5] which tend to focus on urban settings, do not necessarily represent the rest of India. However, human mass migrations almost certainly increase the probability of transmission of ARG across the broader community. We do not suggest pilgrimages are not important, nor do we suggest they should not continue, but results show pilgrimage areas, such as Rishikesh-Haridwar, are potential "hot spots" for AR transmission at large scales. This is particularly true because areas like Rishikesh-Haridwar often have inadequate waste treatment facilities, which are especially overloaded when visitor populations increase; i.e., human populations are greatest when waterborne bla_{NDM-1} levels are highest. Therefore, local officials are urged to consider improving waste treatment facilities in pilgrimage regions to better protect ritual bathing waters. Although full-scale waste treatment plants may not feasible, especially given the transient visiting population, providing greater access to pit privies and providing greater local waste management would reduce bla_{NDM-1} releases into surface waters.

A second major implication of this work is that we have established an ideal site for studying the role of surface waters and sediments on broader AR transmission. We do not yet have comprehensive epidemiological information on bla_{NDM-1} resistant clinical cases in Rishikesh-Haridwar versus national averages, but this is clearly an important next step. We predict that multiresistant bla_{NDM-1} positive clinical pathogens are currently less probable in the Rishikesh-Haridwar region (relative to urban India), but such information might be gathered and used to develop strategies for reducing both the clinical and environmental transmission of mobile genes, like bla_{NDM-1}, to strains of clinical importance.

Although we do not directly link elevated environmental reservoirs of bla_{NDM-1} gene to a change in health in the local population, abundances of such genes appear to be significantly different between Rishikesh-Haridwar residents and visitors from outside the region. Further, we show mass pilgrimages potentially produce "hot spots" for ARG transfer, which may be very influential because exposed visitors return home carrying acquired ARG, both elsewhere in the country and around the world. We do not question pilgrimages because they provide huge social benefit, but waste handling and treatment facilities should be improved in such areas to reduce the probability of broader AR dissemination of mobile ARG like bla_{NDM-1}.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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