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ORIGINAL PAPER

Environmental pollution with antimicrobial agents from bulk drug manufacturing industries in Hyderabad, South India, is associated with dissemination of extended-spectrum beta-lactamase and carbapenemase-producing pathogens

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Abstract
Purpose High antibiotic and antifungal concentrations in wastewater from anti-infective drug production may exert selection pressure for multidrug-resistant (MDR) pathogens. We investigated the environmental presence of active pharmaceutical ingredients and their association with MDR Gram-negative bacteria in Hyderabad, South India, a major production area for the global bulk drug market.
Methods From Nov 19 to 28, 2016, water samples were collected from the direct environment of bulk drug manufacturing facilities, the vicinity of two sewage treatment plants, the Musi River, and habitats in Hyderabad and nearby villages. Samples were analyzed for 25 anti-infective pharmaceuticals with liquid chromatography–tandem mass spectrometry and for MDR Gram-negative bacteria using chromogenic culture media. In addition, specimens were screened with PCR for bla_{VIM}, bla_{KPC}, bla_{NDM}, $bla_{\text{IMP-1}}$, and $bla_{\text{OXA-48}}$ resistance genes.
Results All environmental specimens from 28 different sampling sites were contaminated with antimicrobials. High concentrations of moxifloxacin, voriconazole, and fluconazole (up to 694.1, 2500, and 236,950 µg/L, respectively) as well as increased concentrations of eight other antibiotics were found in sewers in the Patancheru–Bollaram industrial area. Corresponding microbiological analyses revealed an extensive presence of extended-spectrum beta-lactamase and carbapenemase-producing Enterobacteriaceae and non-fermenters (carrying mainly $bla_{\text{OXA-48}}$, bla_{NDM}, and bla_{KPC}) in more than 95% of the samples.
Conclusions Insufficient wastewater management by bulk drug manufacturing facilities leads to unprecedented contamination of water resources with antimicrobial pharmaceuticals, which seems to be associated with the selection and dissemination of carbapenemase-producing pathogens. The development and global spread of antimicrobial resistance present a major challenge for pharmaceutical producers and regulatory agencies.

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Keywords Antibiotics · Antifungal agents · Antimicrobial resistance · Multidrug-resistant (MDR) pathogens · Carbapenemase-producing Enterobacteriaceae (CPE) · Non-fermenters · Colonization · Infection · Selection pressure

Introduction

The rising prevalence of antimicrobial resistance in clinically relevant pathogens exerts enormous pressure on the global human healthcare system and is estimated to cause several hundred thousand deaths annually [1, 2]. While resistance is a naturally occurring phenomenon, the increasing use of anti-infectives since the second half of the 20th century has created artificially strong selection pressure for resistant microorganisms [3]. The global emergence and spread of antibiotic resistance are accelerated by various human behaviors, including inappropriate use of antimicrobial agents, poor infection prevention and control within healthcare systems, insufficient control of antibiotic pollution of the environment, and international travel and food trade [4–11]. To mitigate this threat, it is essential to identify sources and dissemination routes of multidrug-resistant (MDR) bacteria and antibiotic resistance genes [4–6, 15]. Today, the most important tools against the spread of MDR organisms are intensified infection control, surveillance, and antimicrobial stewardship [6].

Low levels (0.1–1 µg/L) of pharmaceuticals, including antibiotics, have been detected in surface, ground, and drinking water worldwide [3, 12, 15]. Incorrect usage and disposal have been identified as the major sources of environmental micro-contamination. The environment around bulk drug manufacturing plants has repeatedly been identified as a source for resistant organisms, especially in India and the People's Republic of China, which supply most of the world's antibiotics [3, 12–14, 16, 17, 20]. However, the extent to which high concentrations of antimicrobial agents in the environment contribute to the development of MDR organisms has not yet been conclusively determined [3]. Unfortunately, current regulatory systems of pharmaceutical production do not address resistance [15]. Several studies have measured environmental concentrations close to or exceeding the minimal inhibitory concentrations (MICs) of certain antibiotics, such as ciprofloxacin, in samples generally linked to pollution from bulk drug production facilities [3, 12–14, 21]. It is well known that antibiotic concentrations below the MICs can select for resistant bacteria [3, 4, 22].

India currently supplies approximately 20% of the world's generic drugs, with US$15 billion in revenue in 2014 [23], and anti-infectives account for a substantial share of the total. Particular bulk drug manufacturing plants in Hyderabad, South India, have been shown to dump waste into their surroundings or fail to treat manufacturing discharges appropriately, resulting in the contamination of rivers and lakes [12–14, 17, 20]. The substantial quantities of antibiotic pollution, combined with runoff from agriculture and human waste, facilitate the growth of MDR bacteria in water bodies and sewage treatment plants [23]. Consequently, India has become a hot spot of drug resistance, with drastic clinical consequences. More than 56,000 newborn babies in India die each year from infections by bacteria that are resistant to first-line antibiotics [24]. The presence of NDM-1 and other carbapenemases in environmental samples has important implications for citizens reliant on public water and sanitation facilities [18, 19].

Microbes' ability to travel within human hosts and traded animals or goods means that multidrug resistance can move around the world within a flight time of only a few hours [11]. Visitors to a country with a high prevalence of antibiotic resistance often return home colonized by MDR bacteria, which are then easily transmitted to others [7–10], including 5–10% of household members [10]. For instance, during travel to India, the specific risk of acquiring Enterobacteriaceae that produce extended-spectrum beta-lactamases (ESBL's) is about 70–90% [8–10].

This study was designed to determine the environmental presence of active anti-infective pharmaceuticals in a major production area for the global bulk drug market. The aim is to document the ongoing environmental pollution by the pharmaceutical industries in Hyderabad, South India, and to highlight its association with the presence of MDR pathogens.

Methods

Setting

Hyderabad is the capital of the southern Indian state of Telangana and occupies approximately 650 km^2 along the banks of the Musi River. Its population was estimated at 10.1 million (with 11.7 million in the metropolitan area) in 2016, making it the fourth most populous city and sixth most populous urban agglomeration in India [25]. Hyderabad is the growing hub for pharmaceutical manufacturing companies in South India. Many companies in Hyderabad are approved by the World Health Organization (WHO), US Food and Drug Administration (FDA), and European authorities for good manufacturing practice (GMP), and have international reputations (e.g., Dr. Reddy's Laboratories, Aurobindo Pharma).

Patancheru–Bollaram is an industrial zone located approximately 32 km outside Hyderabad. In the early 1980s, many bulk drug, chemical, pesticide and other

manufacturing plants were established there. Today, Patancheru–Bollaram and the surrounding villages are home to more than 100 industries, including more than 30 pharmaceutical drug manufacturers (Fig. 1) that supply nearly all leading pharmaceutical companies in the world [23, 26]. Although they generate enormous amounts of industrial effluents every day [27], the pharmaceutical industries in Patancheru–Bollaram are not connected to a functioning water supply or wastewater network (Fig. 2, Supplementary Fig. 1). On-site treatment of wastewater in primary effluent treatment plants includes reverse osmosis, strippers, multiple effect evaporators, and agitated thin-film dryers [26]. Pretreated effluents, low in total dissolved solids, are transported by trucks to a common effluent treatment plant operated by Patancheru Enviro Tech Ltd. (PETL) for further processing (Supplementary Fig. 2). Officially, PETL receives approximately 1600–2000 m^3 of industrial waste per day [27]. Until a few years ago, the effluent from PETL was discharged into the Isakavagu creek, which feeds the Nakkavagu, Manjira, and eventually Godawari rivers [13, 26]. Following a public interest petition in 1997, the Indian Supreme Court ordered the pollution control authorities to channelize effluents from PETL through an 18 km pipeline to the Amberpet mega sewage treatment plant in Hyderabad, so that the effluents could be diluted with sewage [27, 28]. The PETL outlet was connected to the pipeline 12 years later, in July 2009. Since then, the final treated wastewater has been discharged into the Musi River (Supplementary Fig. 3).

Fig. 1 Shepherd with a herd of goats amidst the Patancheru–Bollaram industrial zone

Fig. 2 Polluted sewer, Patancheru–Bollaram industrial zone (when collecting s11)

Sampling

Different sampling sites were chosen to cover the direct vicinity of bulk drug manufacturing facilities, rivers, lakes, ground water, drinking water, water sources contaminated by sewage treatment plants, and surface water from populated urban as well as rural areas. The selection of sites was a matter of availability without claiming to be fully representative. Accurate GPS coordinates and photographic documentation are provided for all sampling sites (Supplementary Fig. 4). An overview map is given in Fig. 3, and a more detailed marked map is available at http://umap.openstreetmap.fr/de/map/samples-hyderabad_123988#11/17.4375/78.4561. Microbiological specimens were collected using ESwabs™ (Copan, Brescia, Italy), a liquid-based multipurpose collection and transport system, and were transferred to the microbiology laboratory in Leipzig, Germany, within 48 h. Water samples destined for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis were transferred to the laboratory in Nuremberg, Germany, within 48 h and frozen at −80 °C. Surface, seepage, and tap water samples are not listed in the current notifications (Exim Policy Schedule 2, http://www.dgft.org/export-policy-schedule-2.html) issued by the India Directorate General of Foreign Trade, so no permit was required for export.

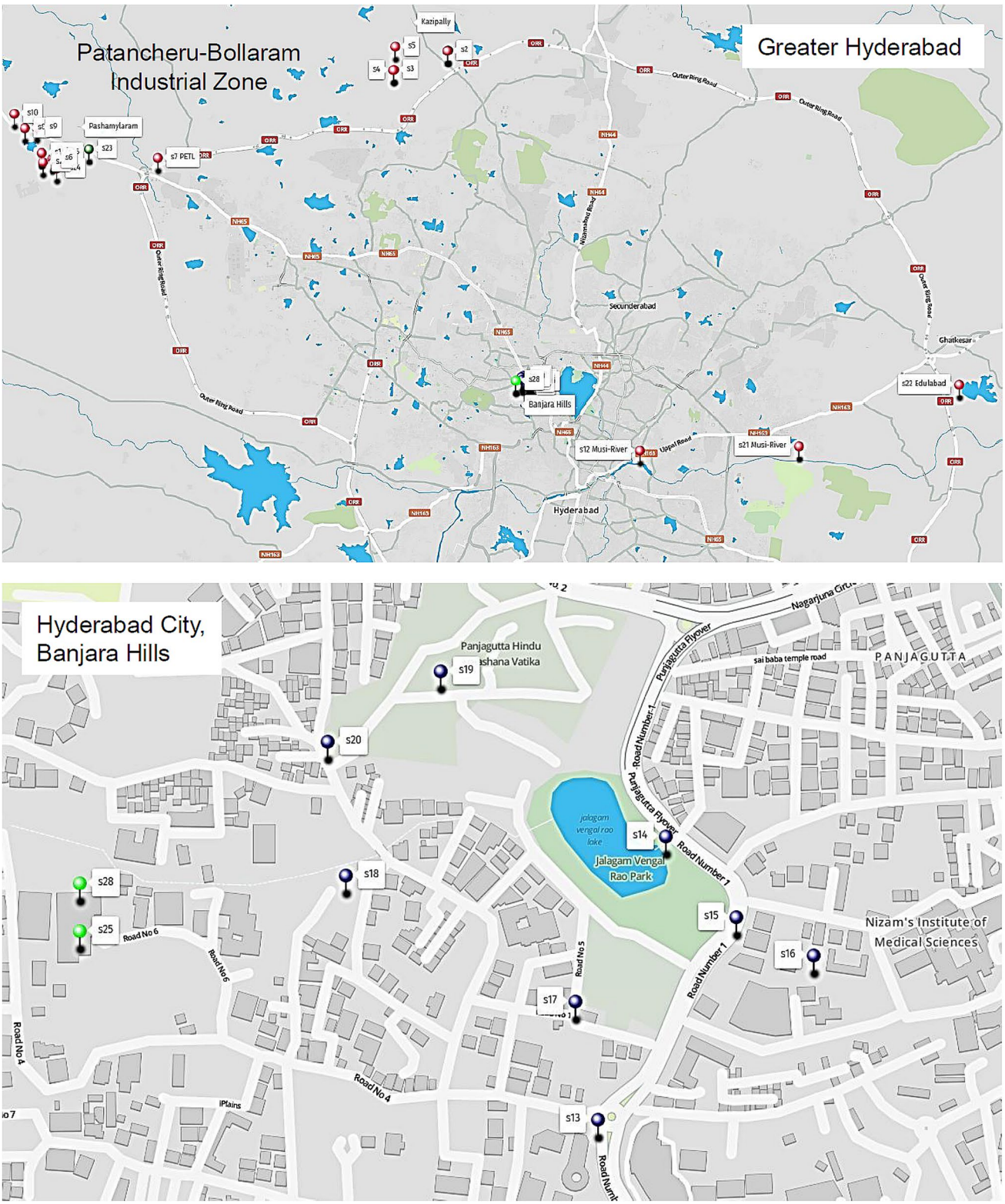

Fig. 3 Map of sampling locations in Hyderabad and surrounding areas. A more detailed marked map is available from http://umap.openstreetmap.fr/de/map/samples-hyderabad_123988#11/17.4375/78.4561

Bacterial cultures and identification of isolates

Microbiological specimens were plated onto two selective culture media plates (CHROMagar™ ESBL and CHROMagar™ KPC; CHROMagar, Paris, France) according to the manufacturer's instructions for the isolation of ESBL and carbapenemase-producing bacteria. Additional cultures were established to determine the total bacterial content of the samples. Three colonies identical in macro-morphology on each selective plate were identified using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (bioMérieux, Marcy l'Etoile, France).

Detection of extended-spectrum beta-lactamases

Bacterial isolates were tested with MIC Test Strips (Liofilchem, Roseto degli Abruzzi, Italy) containing gradients of cefotaxime/cefotaxime + clavulanic acid (CTX/CTL), ceftazidime/ceftazidime + clavulanic acid (CAZ/CAL), and cefepime/cefepime + clavulanic acid (FEP/FEL). Interpretation followed the manufacturer's recommendations. Strains were considered ESBL-positive when three doubling dilutions in the presence of clavulanic acid caused a reduction of the MIC (MIC ratio of $\geq$8).

Detection of carbapenemases

Carbapenemases were detected using the on-demand real-time PCR system Xpert® Carba-R (Cepheid, Sunnyvale, USA), capturing the VIM, IMP-1, NDM, KPC, and OXA-48 variants [29].

Liquid chromatography–tandem mass spectrometry

Samples were analyzed with LC–MS/MS for the presence of 25 anti-infective pharmaceuticals (see Table 2) using ten different methods. The exact methodology is given in Supplementary Text File 1. Levofloxacin was not analyzed in enantioselective mode; thus, concentrations shown for levofloxacin could as well be those of ofloxacin. Samples with concentrations above the upper quantification limit were diluted and analyzed again. For example, the sample with the highest concentration in all samples was diluted 1:5000 with 0.1% formic acid for quantification of fluconazole (Fig. 4).

Statistical analysis

Only descriptive statistics were used. Numerical variables are given as means, and categorical variables are given as frequencies or proportions.

Ethics compliance

This study was performed in accordance with the ethical guidelines of the 1964 Declaration of Helsinki and its later amendments. Since this is an environmental study that does not involve patients, formal consent is not required by the federal legislation of the Free State of Saxony, Germany.

Results

Detection of multidrug-resistant pathogens

The 28 samples can be subdivided into 4 tap water, 1 borehole water, and 23 environmental samples (Table 1). Of the latter, 10 were taken in the direct vicinity of pharmaceutical factories, 4 from rural areas, and 9 from urban sites in Hyderabad.

The only samples that tested negative for all MDR pathogens (s23 and s28) were taken from tap water of a four-star hotel in Banjara Hills, Hyderabad. Tap water from a food stall in the suburb of Dundigal (s1) contained ESBL-producing Enterobacteriaceae and non-fermenting bacteria, including species testing positive for $bla_{\text{OXA-48}}$. Water from a borehole in Dundigal (s2) and tap water from the village of Isnapur (s23) contained Enterobacteriaceae and/or non-fermenters without multidrug resistance.

All 23 environmental samples contained ESBL as well as carbapenemase-producing bacteria (mainly Enterobacteriaceae, but also non-fermenters), of which 22 tested positive for $bla_{\text{OXA-48}}$, 10 for bla_{NDM}, 7 for bla_{KPC}, 5 for bla_{VIM}, and 5 for $bla_{\text{IMP-1}}$. Two samples (s12 and s18), one of which derived from the Musi River, were positive for all tested carbapenemase genes. In the 10 samples from the direct vicinity of bulk drug manufacturing plants, the dominant carbapenemase gene was $bla_{\text{OXA-48}}$ (9 samples), followed by bla_{NDM} (3) and bla_{KPC} (3).

Detection of pharmaceuticals by liquid chromatography–tandem mass spectrometry

Specimens from 16 sampling sites were analyzed (Table 2). The pharmaceutical samples are numbered in the same way as the microbiological samples but are marked with an asterisk.

All environmental samples were found to be contaminated with antifungals and/or antibiotics. Fluconazole was detectable in 13 of the samples, voriconazole in 12, moxifloxacin in 9, linezolid in 8, levofloxacin in 6, clarithromycin in 6, and ciprofloxacin in 5. Ampicillin, doxycycline, trimethoprim, and sulfamethoxazole were also detectable. Samples from sewers in the Patancheru–Bollaram industrial area contained extremely high concentrations of

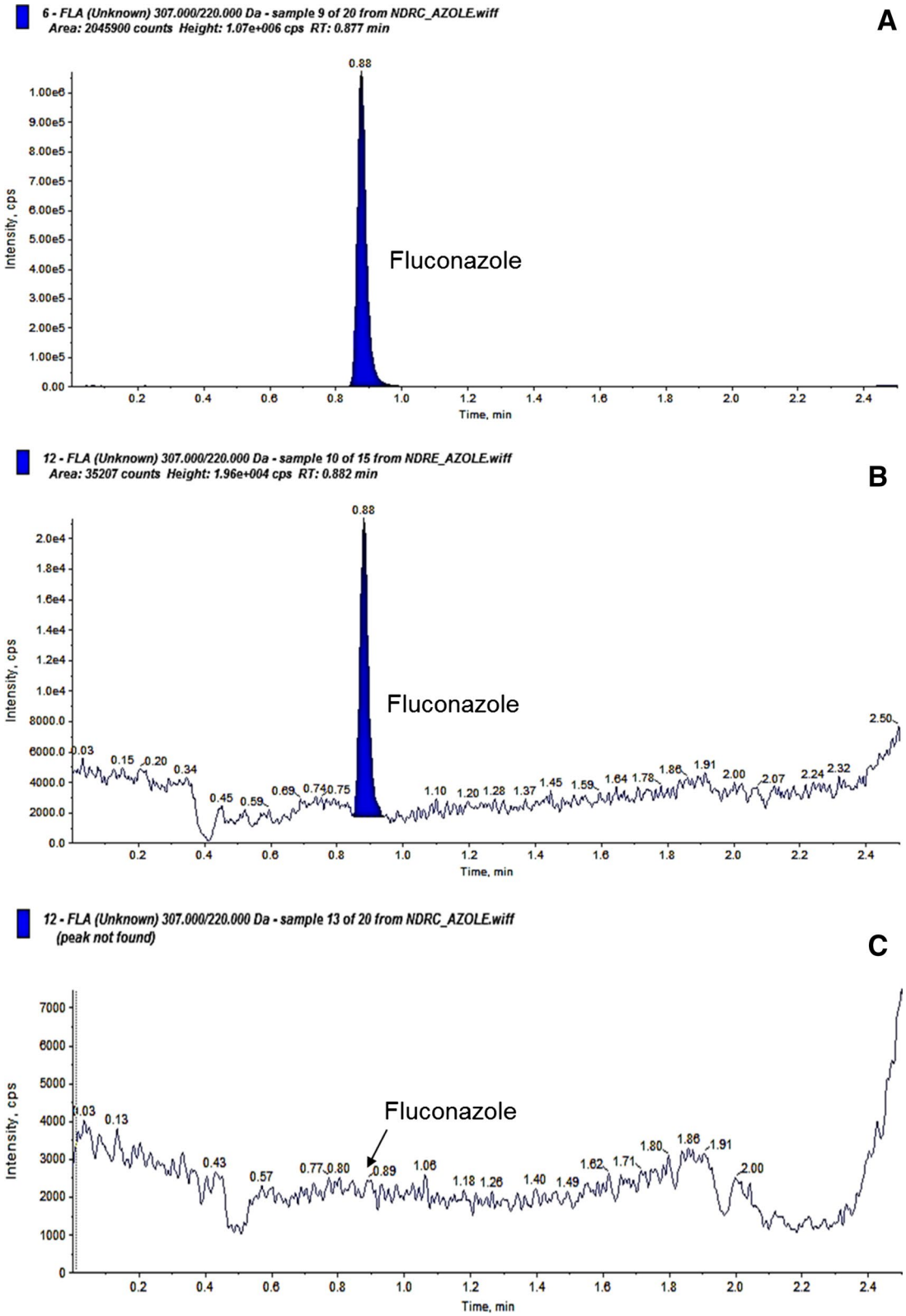

Fig. 4 Chromatograms of samples with detection of fluconazole. *Panel A* shows the highest measured concentration of fluconazole (s6*, 236,950 µg/L) and *Panel B* a comparatively low concentration (s12*, 13.1 µg/L). *Panel C* belongs to a blank sample set at highest sensitivity which contains no fluconazole. The results were verified by analyzing the samples in duplicate

Table 1 Samples with precise geographical location and their microbiological findings

Sample ID	Date of collection	Location	GPS coordinates	Enterobacteriaceae	Non-fermenters	ESBL-production	bla_{VIM}	bla_{KPC}	bla_{NDM}	bla_{IMP-1}	bla_{OXA-48}
s1	19 Nov 2016	Tap water from a food stall, Dundigal	17°35′45″N, 78°24′13″E	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos
s2	19 Nov 2016	Borehole water, Dundigal	17°35′46″N, 78°24′14″E	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
s3	19 Nov 2016	Sewage storage, Patancheru Kazipally industrial area	17°35′09″N, 78°22′26″E	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Pos
s4	19 Nov 2016	Effluent from a sewage storage, Patancheru Kazipally industrial area	17°35′08″N, 78°22′25″E	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos
s5	19 Nov 2016	Kazipally Lake, Patancheru Kazipally industrial area	17°35′54″N, 78°22′29″E	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Pos
s6	19 Nov 2016	Sewer, Patancheru Pashamylaram industrial area	17°32′21″N, 78°11′08″E	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos
s7	20 Nov 2016	Creek near PETL	17°32′23″N, 78°14′38″E	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos
s8	20 Nov 2016	Rudraram Village Lake, Patancheru Pashamylaram industrial area	17°33′19″N, 78°10′14″E	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos
s9	20 Nov 2016	Sewer, Patancheru Pashamylaram industrial area	17°33′20″N, 78°10′37″E	Pos	Pos	Pos	Neg	Pos	Neg	Neg	Neg
s10	20 Nov 2016	Rice field, Rudraram village	17°33′46″N, 78°09′53″E	Pos	Pos	Pos	Pos	Neg	Pos	Neg	Pos
s11	20 Nov 2016	Sewer, Patancheru Pashamylaram industrial area	17°32′32″N, 78°10′47″E	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos
s12	20 Nov 2016	Musi River, Hyderabad City, upstream the Amberpet treatment plant	17°23′08″N, 78°30′36″E	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
s13	21 Nov 2016	Surface water storage tank, Hyderabad City, Banjara Hills (near GVK one shopping mall)	17°25′11″N, 78°26′52″E	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos
s14	21 Nov 2016	Surface water, Jalagam Vengala Rao Park Lake, Hyderabad City, Banjara Hills	17°25′23″N, 78°26′55″E	Pos	Pos	Pos	Pos	Neg	Pos	Neg	Pos

Table 1 continued

Sample ID	Date of collection	Location	GPS coordinates	Enterobacteriaceae	Non-fermenters	ESBL-production	*bla*$_{VIM}$	*bla*$_{KPC}$	*bla*$_{NDM}$	*bla*$_{IMP-1}$	*bla*$_{OXA-48}$
s15	21 Nov 2016	Surface water from a ditch in front of the Nizam Institute of Medical Sciences, Hyderabad City, Banjara Hills	17°25′20″N, 78°26′58″E	Pos	Pos	Pos	Pos	Neg	Pos	Neg	Pos
s16	21 Nov 2016	Small sewer crossing the terrain of the Nizam Institute of Medical Sciences, Hyderabad City, Banjara Hills	17°25′17″N, 78°27′03″E	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos
s17	21 Nov 2016	Surface water from a construction site in Hyderabad City, Banjara Hills	17°25′16″N, 78°26′51″E	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos
s18	21 Nov 2016	Sewer in Hyderabad City, Banjara Hills	17°25′21″N, 78°26′41″E	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
s19	21 Nov 2016	Small sewer crossing the terrain of the Hindu Cemetery, Hyderabad City, Banjara Hills	17°25′30″N, 78°26′45″E	Pos	Pos	Pos	Neg	Neg	Pos	Pos	Pos
s20	21 Nov 2016	Surface water from a street in Hyderabad City, Banjara Hills	17°25′27′′N, 78°26′40′′E	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos
s21	26 Nov 2016	Weir in the Musi River, Hyderabad City, downstream the Amberpet treatment plant	17°23′17″N, 78°35′53″E	Pos	Pos	Pos	Neg	Pos	Pos	Pos	Pos
s22	26 Nov 2016	Irrigation channel near Edulabad village (East of Hyderabad)	17°25′12″N, 78°41′11″E	Pos	Pos	Pos	Neg	Pos	Pos	Neg	Pos
s23	27 Nov 2016	Tap water from the inner courtyard of a house in Isnapur village, near the Patancheru industrial area	17°32′39″N, 78°12′21″E	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg
s24	27 Nov 2016	Surface water from a pond, Patancheru Pashamylaram industrial area	17°32′03″N, 78°11′17″E	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos
s25	28 Nov 2016	Tap water from room number 306, Radisson Blu Hotel Banjara Hills, Hyderabad City	17°25′19″N, 78°26′29″E	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Table 1 continued

Sample ID	Date of collection	Location	GPS coordinates	Enterobacteriaceae	Non-fermenters	ESBL-production	bla_{VIM}	bla_{KPC}	bla_{NDM}	bla_{IMP-1}	bla_{OXA-48}
s26	28 Nov 2016	Sewer, Patancheru industrial area	17°32′32″N, 78°10′47″E	Pos	Pos	Pos	Neg	Pos	Neg	Pos	Pos
s27	28 Nov 2016	Surface water from a pond, Patancheru Pashamylaram industrial area	17°32′14″N, 78°10′50″E	Pos	Pos	Pos	Neg	Pos	Neg	Neg	Pos
s28	28 Nov 2016	Tap water from room number 347, Radisson Blu Hotel Banjara Hills, Hyderabad City	17°25′21″N, 78°26′29″E	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Note that the column "Enterobacteriaceae" summarizes individual pathogens such as *Escherichia coli*, *Klebsiella* spp., and *Enterobacter* spp., and the column "non-fermenting bacteria" includes pathogens such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*

ESBL extended-spectrum beta-lactamase, *GPS* global positioning system, *Neg* negative, *PETL* Patancheru Enviro Tech Ltd., *Pos* positive

fluconazole (s3*, s4*, and s6*; up to 236,950 µg/L) and voriconazole (s9*; up to 2500 µg/L) as well as nine antibiotics (with the highest values measured for moxifloxacin in s26*). A sample from the Musi River (s12*), which represents the final stretch of the wastewater discharge, contained the highest number of different antimicrobial agents (9) along with many resistance genes encoding carbapenemases. In contrast, tap water from villages (s23*) and water from a borehole in Dundigal (s2*) were not contaminated with pharmaceuticals or showed values in the range of the detection limits (s1*).

Compared with the suggested environmental regulation limit (cut-off for resistance selection) [3], moxifloxacin concentrations in the samples were up to 5500 times as high, ciprofloxacin up to 700 times, clarithromycin about 110 times, ampicillin about 115 times, and levofloxacin/ofloxacin about 50 times. The concentration of fluconazole measured in s6* was approximately 950,000 times as high as the proposed limit. This particular sample was repeatedly analyzed by different laboratory approaches, but the result was always within <10% difference to the first analysis.

Discussion

We found carbapenemase-producing Enterobacteriaceae (CPE) and non-fermenters in more than 95% of our samples from Hyderabad, and the proportion of ESBL-producing organisms was 100%. Excessively high concentrations of clinically relevant antibiotics and antifungal agents were also measured in the environment. The most notable finding is the detection of fluconazole at a concentration of 236,950 µg/L (more than 20 times greater than therapeutically desired levels in the blood) in a sewage sample (s6*) from the Patancheru–Bollaram industrial zone. To our knowledge, this is the highest concentration of any drug ever measured in the environment. The uniqueness of this finding may be the result of low water flow, evaporation of water (ambient temperature was 27 °C, leading to more concentrated samples), and discharge of a production lot that may have not met quality criteria. Fluconazole levels from other sampling sites were in a range described by Larsson et al. before [13].

Our findings confirm those of previous studies that demonstrated a strong association between environmentally stable anti-infective residue pollution and the presence of MDR bacteria [12–14, 16, 17, 20, 21]. According to our own Internet-based research, in 2016, more than 40 pharmaceutical factories in Hyderabad produced antimicrobial drugs and/or intermediates, in particular fluoroquinolones, such as ciprofloxacin, levofloxacin, and moxifloxacin, but also various other antibiotics (i.e., linezolid,

Table 2 Detection of active pharmaceutical ingredients in environmental specimens using liquid chromatography-tandem mass spectrometry

Antimicrobial agent (µg/L)	Number of samples tested positive for (%)	Proposed environmental regulation limit (µg/L)	Sample ID (corresponding location with GPS coordinates: see Table 1)															
			S1*	S2*	S3*	S4*	S5*	S6*	S8*	S9*	S11*	S12*	S21*	S22*	S23*	S24*	S26*	S27*
Fluconazole	13 (81.3)	0.25	N/D	N/D	24,007	48,311	1753	**236,950**	261.5	37.1	199.8	13.1	37.1	18.5	N/D	1331	243.8	147.3
Voriconazole	12 (75.0)	N/A	N/D	N/D	306.4	324.9	4.3	5.0	1.5	**2500**	4.0	1.7	24.5	45.4	N/D	N/D	6.2	8.0
Moxifloxacin	9 (56.3)	0.125	BDL	N/D	31.7	7.1	8.3	29.5	N/D	N/D	279.4	BDL	N/D	N/D	N/D	N/D	**694.1**	BDL
Linezolid	8 (50.0)	8	BDL	N/D	**37.0**	13.6	BDL	N/D	N/D	N/D	N/D	6.7	8.5	5.4	N/D	N/D	N/D	N/D
Levofloxacin[a]	6 (37.5)	0.25	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	2.1	**12.8**	10.0	4.6	N/D	N/D	2.2	N/D
Clarithromycin	6 (37.5)	0.25	N/D	N/D	N/D	N/D	N/D	13.5	N/D	N/D	N/D	BDL	**27.7**	13.3	N/D	BDL	N/D	N/D
Ciprofloxacin	5 (31.3)	0.064	BDL	N/D	N/D	N/D	NN	19.4	N/D	N/D	N/D	40.1	**44.7**	BDL	N/D	N/D	N/D	N/D
Trimethoprim	5 (31.3)	0.5	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	BDL	BDL	BDL	N/D	N/D	N/D	N/D
Sulfamethoxazole	4 (25.0)	16	N/D	N/D	N/D	N/D	N/D	BDL	N/D	N/D	N/D	BDL	**10.6**	BDL	N/D	N/D	N/D	N/D
Ampicillin	3 (18.8)	0.25	N/D	N/D	N/D	N/D	N/D	BDL	N/D	N/D	BDL	N/D	N/D	N/D	N/D	N/D	**29.1**	N/D
Doxycycline	1 (6.3)	2	N/D	N/D	N/D	N/D	N/D	N/D	N/D	**14.9**	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
No. of proven antimicrobials in the same sample			3	0	4	4	4	7	2	3	5	9	8	8	0	2	5	3

The highest value of individual antimicrobials is given in bold print. Environmental regulation limits (cut-off for resistance selection) suggested by Bengsson-Palme and Larsson in 2015 [3] are given in the third column. Amoxicillin, anidulafungin, caspofungin, cefazolin, ceftazidime, cefuroxime, clavulanic acid, flucloxacillin, meropenem, penicillin G, piperacillin, tazobactam, posaconazole, and isavuconazole were not detectable in any of the tested samples

Detection limits Fluconazole 2.59 µg/L, voriconazole 1.26 µg/L, moxifloxacin 5.98 µg/L, linezolid 5.31 µg/L, levofloxacin 2.03 µg/L, clarithromycin 12.7 µg/L, ciprofloxacin 4.96 µg/L, trimethoprim 7.53 µg/L, sulfamethoxazole 9.02 µg/L, ampicillin 12.7 µg/L, doxycycline 2.32 µg/L, amoxicillin 4.59 µg/L, anidulafungin 0.769 µg/L, caspofungin 0.798 µg/L, cefazolin 28.2 µg/L, ceftazidime 30.2 µg/L, cefuroxime 11.1 µg/L, clavulanic acid 1.90 µg/L, flucloxacillin 15.1 µg/L, meropenem 12.7 µg/L, penicillin G 25.9 µg/L, piperacillin 18.6 µg/L, tazobactam 68.7 µg/L, posaconazole 0.695 µg/L, isavuconazole 1.77 µg/L

BDL = traces, but below the detection limit, *N/A* not applicable, *N/D* not detectable

[a] Levofloxacin was not analyzed in enantioselective mode; thus, concentrations shown for levofloxacin could as well be those of ofloxacin

clarithromycin, trimethoprim, sulfamethoxazole, doxycycline, ampicillin, piperacillin, tazobactam, and meropenem) and antifungal agents (i.e., fluconazole and voriconazole). The proportion of proven contaminations with antifungal agents were higher than that with antibiotics, which might reflect the manufacturing procedures at the time. This assumption is supported by the data of concentration measurement with LC–MS/MS which suggest that at the time of sampling (or shortly before), fluconazole and voriconazole were synthesized and discharged. Notably, agents such as fluconazole, voriconazole, or moxifloxacin are synthesized in a traditional chemical way with no natural nucleus, and therefore, they are detectable in the environment for longer time periods. Consequentially, compounds which are made from biological origin (e.g., beta-lactam antibiotics) were not present in reasonably quantifiable concentrations in our analyses.

India is a region of high prevalence of MDR organisms with a substantial potential of spread to other regions of the world [18, 30]. According to the Delhi Neonatal Infection Study, in the period 2011–2014, high rates of multidrug resistance were observed in all clinically relevant pathogens, with 82% of *Acinetobacter* isolates, 54% of *Klebsiella* isolates, and 38% of *Escherichia coli* isolates, leading to case fatality rates of 40–60% [24]. Methicillin resistance was detected in 61% of coagulase-negative staphylococci and 38% of *Staphylococcus aureus* isolates [24]. In another study from New Delhi, ESBL carriage of breast-fed neonates increased threefold from day 1 to 60; the reservoirs for these genes are most likely linked to the mother and environment [31]. A study from Mumbai revealed that 51.9% of patients admitted to the intensive care unit of a tertiary care facility carried CPE in their guts [32]. Antibiotic stewardship efforts in Indian hospitals are still incipient [33].

A report published for the 2015 World Toilet Day stated that in the world's second most populous nation, 60.4% of Indians do not have access to safe and private toilets [34]. MDR bacteria are integrated into the human intestinal microbiome/resistome and may stay there for a long period [4], further complicating the situation. MDR Enterobacteriaceae, such as *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Klebsiella pneumoniae*, may have immediate life-threatening effects during outbreaks in hospitals, especially for high-risk populations such as transplant recipients [35]. Moreover, affected patients may be colonized for several years [36], and the risk of infection due to intestinal colonization with *Klebsiella* strains is at least 5% [37].

Since antibiotic resistance and the associated genes are ubiquitous and ancient (e.g., ESBL and fluoroquinolone resistance genes, such as *qnr*) [1, 3, 7, 14], their rapid spread in recent years must be attributed to modern human behavior and its influence on the environmental resistome [4–7, 30]. This risk can be reduced through improved management of waste containing antibiotic residues and antibiotic-resistant microorganisms [3, 20, 22, 38, 39].

Currently, Hyderabad accounts for approximately 40% of the total Indian bulk drug production and 50% of the bulk drug exports [23]. The pharmaceutical industries and their exports are expected to grow 20% annually. Despite decades of campaigning by local NGOs and legal action taken to the highest Indian courts, the pollution of the surroundings of manufacturing plants has not been reduced [23, 28]. In fact, regulation targeting the pharmaceutical industry is actually becoming more relaxed as the government lifts restrictions on plant expansion and introduces changes to the national pollution index [23]. This index, which has been in place since 2009, has repeatedly classified the Patancheru–Bollaram industrial area as "critically polluted" [26, 27]. The government recently removed certain criteria relating to health and the environment from the index in the name of simplification, despite heavy criticism by the media that these changes were made to benefit polluting industries [23, 28]. Although the Supreme Court demanded that the industries ensure "zero liquid discharge," which means that they would have to effectively treat their wastewater and reuse it [27, 28], massive violations have reportedly occurred [23, 28]. Since the manufacturing units discharge effluents with different chemical compositions, they need to employ various specialized technologies to ensure zero liquid discharge. As such technologies are expensive, the industries often clandestinely send their effluents directly to PETL or simply drain them into the environment [23, 28]. Since the installation of the Patancheru–Amberpet pipeline, the quality of local rivers around PETL has improved, but the pollution has actually been transferred to the Musi River, which flows through the center of Hyderabad and reaches more than 100 villages in its drainage basin [28]. The main problem is that the Amberpet mega sewage treatment plant is ill equipped to treat pharmaceutical effluents with different chemical compositions, so it simply discharges them into the Musi River [26–28]. A study published in September 2016 showed that concentrations of antibiotics in the Musi River (Supplementary Fig. 5) were 1000 times higher than those usually found in rivers in developed countries [40].

Strength and limitations

The strength of this study is the wide range of sampling sites, accurate documentation of sites, and use of highly sensitive LC–MS/MS and PCR techniques. On the other

hand, we lack a strong control group, and our findings do not provide evidence as to whether the wide spread of carbapenemases in the environment has a direct relationship to antibiotic pollution, since many of the causative genes are present in relatively large proportions of fecal bacteria in India. Each environmental sample contains billions of bacteria. Since PCR was used to determine presence or absence of carbapenemases in the samples, a positive result for all or almost all of these genes in any specimen containing sewage or fecal matter is expected. For example, sewage samples from treatment plants in Sweden likely also contain several carbapenemase genes [22], even though only approximately 200 cases of human CPE infections have been documented in the entire country of Sweden [Joakim Larsson, personal communication]. Therefore, detection of CPE-containing bacteria in an environmental swab has very different implications from the same result in a targeted fecal swab.

Conclusions

Environmental pollution and insufficient wastewater management in one of the world's largest centers for bulk drug production lead to unprecedented antimicrobial drug contamination of surface, ground, and drinking water, which seems to be associated with the selection and spread of carbapenem-resistant Enterobacteriaceae and non-fermenters, such as *Acinetobacter baumannii*. The presence of ESBL and carbapenemase-producing pathogens in environmental samples from the Hyderabad metropolitan area has important implications for people in the city and surrounding countryside who are reliant on public water and sanitation facilities.

Europe has a duty to help mitigate the pollution in Hyderabad and other locations. Regulations must be imposed on the manufacturing process of finished drugs as well as active pharmaceutical ingredients to require strict compliance with environmental laws, adequate modernization of manufacturing units and treatment plants, and international labeling of the origin of medicines in a manner clearly visible for pharmacists, physicians, and consumers.

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Compliance with ethical standards

Conflict of interest All authors deny any potential conflicts of interest.

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