Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study

Timothy R Walsh, Janis Weeks, David M Livermore, Mark A Toleman

Summary

Background Not all patients infected with NDM-1-positive bacteria have a history of hospital admission in India, and extended-spectrum β-lactamases are known to be circulating in the Indian community. We therefore measured the prevalence of the NDM-1 gene in drinking water and seepage samples in New Delhi.

Methods Swabs absorbing about 100 µL of seepage water (ie, water pools in streets or rivulets) and 15 mL samples of public tap water were collected from sites within a 12 km radius of central New Delhi, with each site photographed and documented. Samples were transported to the UK and tested for the presence of the NDM-1 gene, bla_{NDM-1}, by PCR and DNA probing. As a control group, 100 µL sewage effluent samples were taken from the Cardiff Wastewater Treatment Works, Tremorfa, Wales. Bacteria from all samples were recovered and examined for bla_{NDM-1} by PCR and sequencing. We identified NDM-1-positive isolates, undertook susceptibility testing, and, where appropriate, typed the isolates. We undertook Inc typing on bla_{NDM-1}-positive plasmids. Transconjugants were created to assess plasmid transfer frequency and its relation to temperature.

Findings From Sept 26 to Oct 10, 2010, 171 seepage samples and 50 tap water samples from New Delhi and 70 sewage effluent samples from Cardiff Wastewater Treatment Works were collected. We detected bla_{NDM-1} in two of 50 drinking-water samples and 51 of 171 seepage samples from New Delhi; the gene was not found in any sample from Cardiff. Bacteria with bla_{NDM-1} were grown from 12 of 171 seepage samples and two of 50 water samples, and included 11 species in which NDM-1 has not previously been reported, including *Shigella boydii* and *Vibrio cholerae*. Carriage by enterobacteria, aeromonads, and *V cholera* was stable, generally transmissible, and associated with resistance patterns typical for NDM-1; carriage by non-fermenters was unstable in many cases and not associated with typical resistance. 20 strains of bacteria were found in the samples, 12 of which carried bla_{NDM-1} on plasmids, which ranged in size from 140 to 400 kb. Isolates of *Aeromonas caviae* and *V cholerae* carried bla_{NDM-1} on chromosomes. Conjugative transfer was more common at 30°C than at 25°C or 37°C.

Interpretation The presence of NDM-1 β-lactamase-producing bacteria in environmental samples in New Delhi has important implications for people living in the city who are reliant on public water and sanitation facilities. International surveillance of resistance, incorporating environmental sampling as well as examination of clinical isolates, needs to be established as a priority.

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Department of Infection, Immunity and Biochemistry, School of Medicine, Cardiff University, Heath Park, Cardiff, UK (Prof T R Walsh PhD, J Weeks BSc, M A Toleman PhD); **University of Queensland Centre for Clinical Research, University of Queensland, Brisbane, Australia** (Prof T R Walsh); **and Health Protection Agency Microbiology Services, Colindale, London, UK** (D M Livermore PhD)

Correspondence to:
Prof Timothy R Walsh, Centre for Clinical Research (UQCCR) I Level 8, Building 71/918 Royal Brisbane Hospital, Herston QLD 4006, Australia
t.r.walsh@uq.edu.au

Introduction

The spread of mobile carbapenemases among bacterial pathogens is of great concern, not only because these enzymes confer resistance to carbapenems and other β-lactam antibiotics, but also because such pathogens typically are resistant to multiple other antibiotic classes, leaving very few treatment options available.[1,2] This concern is certainly warranted for Enterobacteriaceae that produce NDM-1 β-lactamase.[3] Plasmids carrying the gene for this carbapenemase, bla_{NDM-1}, can have up to 14 other antibiotic resistance determinants (M A Toleman, unpublished data) and can transfer this resistance to other bacteria, resulting in multidrug-resistant or extreme drug-resistant phenotypes.[4] Resistance of this scale could have serious public health implications because so much of modern medicine is dependent on the ability to treat infection.[2,5,6]

So far, the NDM-1 enzyme has been found clinically in Enterobacteriaceae and *Acinetobacter baumannii*,[7] and both our previous study[4] and findings by Deshpande and colleagues[8] showed that NDM-1 is widely disseminated in the Indian subcontinent. The first NDM-1-positive isolate described was from a Swedish resident of Indian origin who contracted a *Klebsiella pneumoniae* infection while in India in late 2007. The patient also had an NDM-1-positive *Escherichia coli* strain in his gut flora.[3] A recent surveillance study[9] showed that isolates containing NDM-1 were circulating in New Delhi as early as 2006, 2 years before the first European case was identified. Since 2008, there has been repeated import of NDM-1-positive bacteria from the Indian subcontinent to Europe, the USA, Canada, Asia, and Australasia, which was often mediated via transfers of patients, as well as some direct transmission in Europe and some unaccounted clusters linked to the Balkans.[10]

Many of the patients from Europe, North America, Asia, and Australasia had been admitted to hospital in India, Pakistan, or Bangladesh, but a few had a history of travel to the subcontinent without hospital admission. For example, some individuals had gut colonisation with producer strains found coincidentally while being assessed for traveller's diarrhoea.[11] Also, the known problem of poor sanitation in some parts of India[12] and the fact that many travellers to the Indian subcontinent experience gut colonisation with other multiresistant bacteria[13] led us to investigate the extent to which bla_{NDM-1} is circulating in community waste seepage and tap water in urban New Delhi and to assess the transmissibility of bla_{NDM-1}-encoding elements.

Methods

Sample collection

An employee of Channel 4 News who holds a UK biology degree oversaw the sample collection and was given strict verbal and written instructions on collection of samples. The corresponding author spoke to the team several times to ensure that proper procedures were followed. Swabs of seepage water and tap water samples from individual sites, picked by team members, within a 12 km radius of central New Delhi were collected. Swabs were taken from waste seepage sites (ie, water pools in streets or rivulets) and absorbed about 100 µL of liquid. Swabs were transported in charcoal agar to preserve bacteria and DNA. 15 mL samples of tap water were taken from public water supplies that local residents use for drinking, washing, and food preparation. The sources and times of all samples were recorded on large-scale maps and photographs of each site were taken. Samples were transported to the UK in airtight UN-approved sealed containers via a personal courier. Seepage water and tap water samples are not listed in the DGFT Notification number 27/2007 issued by the Government of India, Ministry of Commerce and Industry, Department of Commerce, and therefore no permit is required for export. As a control group, we took 100 µL sewage effluent samples from the Cardiff Wastewater Treatment Works, Tremorfa, Wales.

For the **DGFT Notification** see http://www.dgft.org/

Procedures

Swabs were squeezed out in 0·5 mL volumes of sterile molecular grade water (Sigma Aldrich, Poole, UK) in Eppendorf tubes and centrifuged at 3000 *g* for 30 s to remove residual charcoal agar. bla_{NDM-1} was detected by PCR amplification with 1 µL of the swab suspension as a DNA template. We also used in-gel DNA hybridisation with a bla_{NDM-1} probe, as previously described,[4] with the modification that PCR annealing was done at 68°C and the reaction continued for 40 cycles. Resuspended swab samples (in molecular grade water) were also spotted directly onto a hybridisation membrane and probed with bla_{NDM-1}. We assayed water samples both as obtained and after any bacteria had been concentrated 15-fold by centrifugation at 12 000 *g* for 15 min and resuspended in 1 mL of sterile molecular grade water. PCR analysis and hybridisation was then done as for the swab samples. We used *K pneumoniae* IR25-P and K15-D6-37 as NDM-1-positive controls.[4]

To isolate and characterise the phenotypes of NDM-1-positive bacteria, swabs and 50 µL of concentrated tap water were repeatedly spread onto Mueller-Hinton agar (Becton Dickinson, Oxford, UK) containing 100 mg/L vancomycin plus 20 mg/L cefotaxime or 100 mg/L vancomycin plus 0·5 mg/L meropenem until single colonies were obtained. Colonies that grew during overnight incubation (37°C) were selected on the basis of morphology and were transferred with sterile toothpicks onto Mueller-Hinton media containing 0·5 mg/L meropenem. These colonies were screened by use of colony DNA hybridisation and PCR analysis as previously described.[4] All PCR products were confirmed by sequencing.

Positive samples were subcultured onto MacConkey agar (Oxoid Thermofisher, Basingstoke, UK) containing 0·5 mg/L meropenem to ensure culture purity and were identified with the Phoenix automated identification system (Becton Dickinson) or with API 20E strips (bioMérieux, Basingstoke, UK). For samples with putative NDM-1 positive *Vibrio cholerae* and *Shigella* spp, we used strain-specific antisera and species-specific PCR typing.[14] We calculated minimum inhibitory concentrations by micro-broth dilution (Phoenix, Becton Dickinson) or Etest (bioMérieux). NDM-1-positive isolates were sloped and frozen at –80°C. We used *K pneumoniae* IR25-P and K15-D6-37 as positive controls and *E coli* J53 as a negative control.[4]

If several NDM-1-positive isolates of the same species were isolated from one sample, genomic DNA was prepared in agarose blocks and digested with the restriction enzyme *Xba*I (Roche Diagnostics, Mannheim, Germany). We separated DNA fragments by pulsed-field gel electrophoresis on a CHEF-DR III apparatus (Bio-Rad, Hercules, CA, USA) for 20 h at 6 V/cm at 8°C, with initial and final pulse times of 0·5 s and 45 s, respectively.

Transfer of bla_{NDM-1} plasmids from the environmental isolates was done with the following recipients, all handled under appropriate safety conditions: the standard *E coli* J53 azide resistant strain; a clinical strain of *Shigella sonnei* (provided by the Special Antimicrobial Chemotherapy Unit [SACU], NHS Wales); and a clinical strain of *Salmonella enterica* serotype enteritidis (*S enteritidis*; also provided by SACU). We made suspensions of overnight cultures, with the recipient at about one-tenth of the cell density of the donor. About 10^8 colony-forming units of the donor and 10^7 colony-forming units of the recipient were then added to 1 mL of nutrient broth (Becton Dickinson) and left overnight at 25°C, 30°C, or 37°C before being diluted in physiological saline (10^{-3} and 10^{-5}) and plated onto selective media. Conjugation was also attempted on

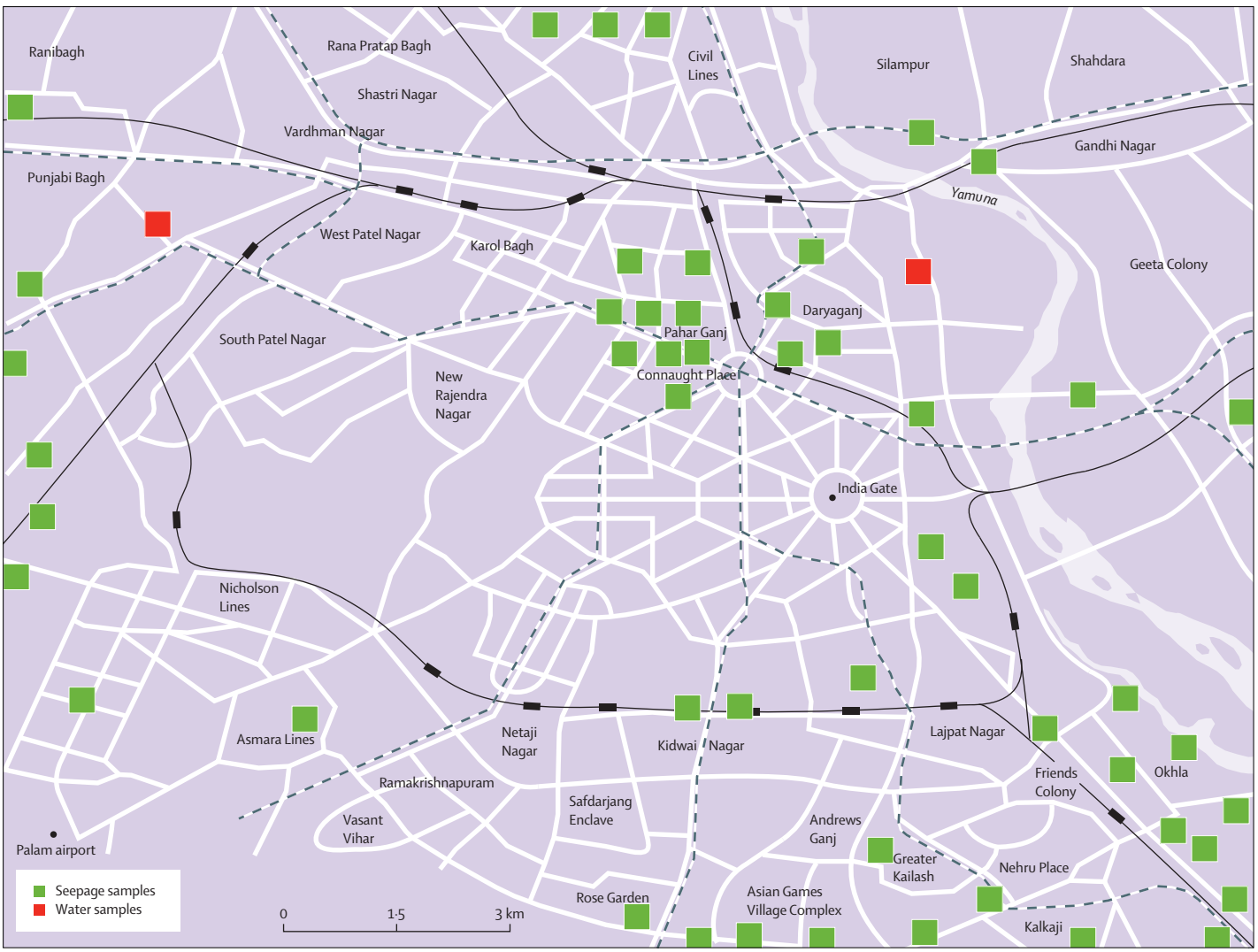

Figure 1: **Map of NDM-1-positive samples from New Delhi centre and surrounding areas**

nutrient agar by adding a defined inoculum (10^8) of the donor directly onto the recipient and incubating overnight at 25°C, 30°C, or 37°C. After 18 h of incubation, the cells were harvested from the agar surface, suspended in 10 mL of water, and diluted in physiological saline (10^{-3}, 10^{-5}, and 10^{-7}). In all cases, the donor and recipient suspensions were also diluted in physiological saline (10^{-5}) and plated onto MacConkey agar (Oxoid, Basingstoke, UK) to confirm purity and to assess the colony count.

We used the following media to recover NDM-1-positive transconjugants: MacConkey agar containing 100 mg/L sodium azide and 0·5 mg/L meropenem for *E coli* J53 transconjugants; MacConkey agar containing 0·5 mg/L meropenem (in this case only lactose fermenters were used as bla_{NDM-1} donors) for *S sonnei* transconjugants; and XLD medium (Oxoid) containing 0·5 mg/L meropenem for *S enteritidis* transconjugants. We confirmed putative transconjugants by biochemical profiling and by detection of bla_{NDM-1} with PCR and DNA hybridisation, as described previously. Plasmids were isolated and typed on the basis of their origin or origins of replication by in-gel hybridisation, as previously described.[4]

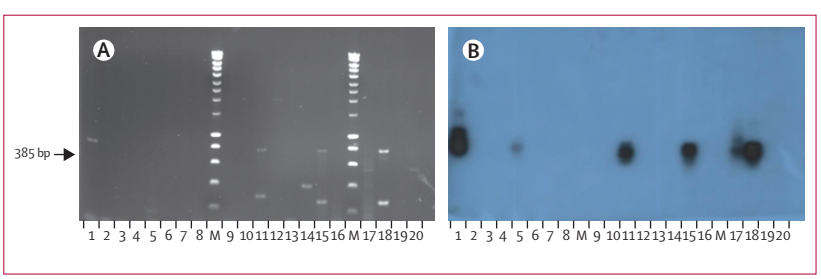

Figure 2: **PCR screening and hybridisation of selected seepage samples**
(A) PCR screening from swabs of seepage samples. Negative and positive controls were run with each batch (data not shown). All corresponding gels were probed with bla_{NDM-1} (B). Lane 5 does not show a PCR product but the increased sensitivity of Southern hybridisation detects low copy numbers of bla_{NDM-1}. PCR products were confirmed by sequencing. M=DNA size marker.

	Species	Minimal inhibitory concentration (mg/L)												Typical bla_{NDM-1} antibiogram	Genetic location	Plasmid		
		CTX	CTZ	IMP	MER	ATM	GEN	AMI	TOB	CIP	FOS	TIG	COL			Size	Stability*	Type
From waste seepage																		
B-3-2	*Pseudomonas putida*	64	4	0·5	2	64	0·25	1	0·25	0·125	256	4	0·125	No	Plasmid	ND	No	..
1-19	*Pseudomonas pseudoalcaligenes*	64	64	2	4	32	2	1	4	16	16	2	0·25	No	Plasmid	ND	No	..
3-1	*Escherichia coli*	512	256	16	32	64	8	4	16	32	16	4	0·5	Yes	Plasmid	140 kb	Yes	A/C
21-9	*Pseudomonas oryzihabitans*	16	4	2	2	16	0·25	2	0·25	0·25	4	4	0·25	No	Plasmid	ND	No	..
25-4	*Klebsiella pneumoniae*	512	256	32	128	64	32	64	16	32	256	8	0·25	Yes	Plasmid	140 kb	Yes	..
33-5	*Escherichia coli*	256	256	64	64	64	16	32	64	32	2	0·5	0·125	Yes	Plasmid	140 kb	Yes	A/C
65-4	*Escherichia coli*	256	128	8	64	32	16	2	32	16	16	0·5	0·125	Yes	Plasmid	140 kb	Yes	..
65-5	*Shigella boydii*	512	512	4	16	256	32	16	8	64	2	4	1	Yes	Plasmid	250 kb	Yes	..
72-28	*Sutonella indologenes*	32	4	2	4	32	1	2	0·5	0·25	>1024	8	2	No	Plasmid	..	No	..
79-6	*Pseudomonas pseudoalcaligenes*	128	16	2	4	32	4	2	2	8	16	8	0·25	No	Plasmid	280 kb	Yes	..
107-5	*Aeromonas caviae*	64	32	16	8	8	8	2	8	16	128	8	0·25	Yes	Chromo	..	Yes	..
107-7	*Pseudomonas putida*	64	1	32	4	0·25	16	16	32	16	256	16	0·25	No	Plasmid	250 kb	Yes	..
116-4	*Stenotrophomonas maltophilia*	256	256	128	64	64	32	64	16	64	256	16	0·5	Yes	Plasmid	250 kb	Yes	..
116-14	*Vibrio cholerae*	>256	>256	8	8	2	1	8	2	2	64	0·5	8	Yes	Plasmid and chromo	400 kb	Yes	..
116-17	*Vibrio cholerae*	>256	>256	16	1	2	1	0·5	2	2	64	0·5	8	Yes	Plasmid	170 kb	Yes	A/C
117-4	*Citrobacter freundii*	128	128	64	128	64	32	64	32	32	4	2	0·5	Yes	Plasmid	140 kb	Yes	A/C
From tap water																		
W32-17	*Achromobacter* spp	256	256	4	4	64	32	16	32	32	32	0·5	0·125	No	Plasmid	ND	No	..
W38-14	*Kingella denitrificans*	32	32	4	16	8	8	2	1	4	4	1	0·5	No	Plasmid	ND	No	..
W38-16	*Achromobacter* spp	128	128	4	2	32	32	16	4	16	32	0·5	0·25	No	Plasmid	ND	No	..
W38-17	*Pseudomonas aeruginosa*	256	256	32	32	16	32	64	32	16	256	8	0·5	Yes	Plasmid	ND	No	..

CTX=cefotaxime. CTZ=ceftazidime. IMP=imipenem. MER=meropenem. ATM=aztreonam. GEN=gentamicin. AMI=amikacin. TOB=tobramycin. CIP=ciprofloxacin. FOS=fosfomycin. TIG=tigecycline. COL=colistin. ND=not determined. Chromo=chromosome. *Plasmids were deemed unstable if lost within a 48-h period during subculturing without antibiotic selection.

Table 1: **Minimal inhibitory concentration of antimicrobials and genetic characteristics of NDM-1-positive bacteria**

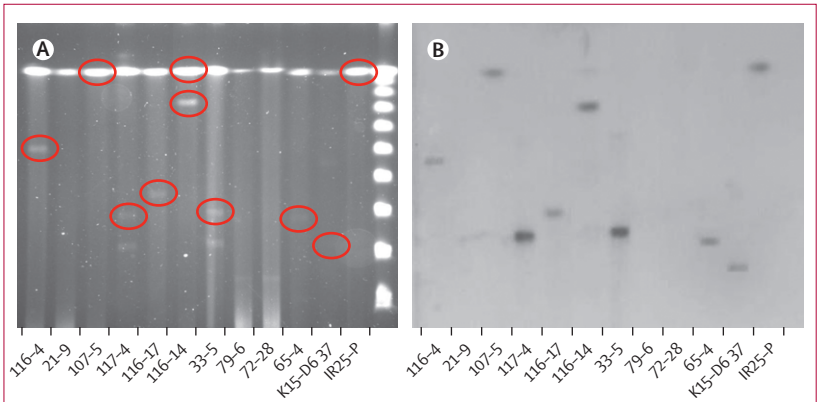

Figure 3: **Hybridisation results for NDM-1-positive isolates**
(A) S1 nuclease pulsed-field gel electrophoresis and (B) corresponding Southern hybridisation blot on some NDM-1-positive strains after 48 h of passaging in antibiotic-free media. K15-D6-37 (120 kb plasmid) was used as a positive control. Isolates 21-9, 79-6, and 72-28 had unstable plasmids that were lost on subculturing after 48 h.

We assessed plasmid stability by serial passage of NDM-1-positive isolates on antibiotic-free and 0·5 mg/L meropenem-containing Mueller-Hinton media (Becton Dickinson). Carriage of bla_{NDM-1} was assessed as previously described.[4] Plasmids were deemed unstable if they were lost after two consecutive passages.

Genomic DNA in agarose blocks was digested with the endonuclease S1 (Invitrogen, Abingdon, UK), and DNA fragments were separated by pulsed-field gel electrophoresis, as described earlier. We then did in-gel hybridisation with a bla_{NDM-1} ^{32}P-labelled probe (Strategene, Amsterdam, Netherlands) with a random-primer method, as previously described.[3] Confirmation of A/C plasmids carrying bla_{NDM-1} was done by hybridisation with IncA/C probes, as previously described.[4] We used *K pneumoniae* IR25-P and K15-D6-37 as positive controls and *E coli* J53 as a negative control.[4]

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author, at all times, had full access to all the data in the study and had final responsibility for the decision to submit for publication.

	Species	Frequency of transfer								
		E coli J53			*S enteritidis* R08/R10			*S sonnei* Q96/Q97		
		25°C	30°C	37°C	25°C	30°C	37°C	25°C	30°C	37°C
3-1	*Escherichia coli*	$4{\cdot}3\times10^{-7}$	$1{\cdot}8\times10^{-4}$	$9{\cdot}1\times10^{-7}$	$<10^{-9}$	$7{\cdot}2\times10^{-6}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$
25-4	*Klebsiella pneumoniae*	$8{\cdot}1\times10^{-7}$	$2{\cdot}7\times10^{-3}$	$<1\times10^{-9}$	$1{\cdot}7\times10^{-7}$	$1{\cdot}5\times10^{-5}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$2{\cdot}2\times10^{-6}$	$<1\times10^{-9}$
33-5	*Escherichia coli*	$1{\cdot}1\times10^{-6}$	$9{\cdot}7\times10^{-6}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$
65-4	*Escherichia coli*	$2{\cdot}1\times10^{-6}$	$5{\cdot}6\times10^{-4}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$1{\cdot}3\times10^{-7}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$
65-5	*Shigella boydii*	$<1\times10^{-9}$	$1{\cdot}9\times10^{-4}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$4{\cdot}1\times10^{-5}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$
79-6	*Pseudomonas pseudoalcaligenes*	$<1\times10^{-9}$	$1{\cdot}1\times10^{-6}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$
107-5	*Aeromonas caviae*	$3{\cdot}3\times10^{-6}$	$7{\cdot}3\times10^{-6}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$< 1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$
107-7	*Pseudomonas putida*	$3{\cdot}5\times10^{-7}$	$2{\cdot}9\times10^{-3}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$< 1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$
116-4	*Stenotrophomonas maltophilia*	$1{\cdot}5\times10^{-5}$	$2{\cdot}5\times10^{-4}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$< 1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$
116-14	*Vibrio cholerae*	$5{\cdot}5\times10^{-6}$	$8{\cdot}7\times10^{-4}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$1{\cdot}6\times10^{-6}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$
116-17	*V cholerae*	$2{\cdot}1\times10^{-6}$	$2{\cdot}5\times10^{-4}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$8{\cdot}7\times10^{-7}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$<1\times10^{-9}$
117-4	*Citrobacter freundii*	$6{\cdot}7\times10^{-5}$	$6{\cdot}7\times10^{-4}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$2{\cdot}1\times10^{-5}$	$<1\times10^{-9}$	$<1\times10^{-9}$	$3{\cdot}1\times10^{-7}$	$<1\times10^{-9}$

Table 2: **Effects of different temperatures on transfer of NDM-1-encoding plasmids from environmental bacteria strains to *Escherichia coli*, *Salmonella enterica* serotype enteritidis, and *Shigella sonnei***

Results

From Sept 26 to Oct 10, 2010, 171 swabs of seepage water and 50 tap water samples from New Delhi and 70 sewage effluent samples from Cardiff Wastewater Treatment Works were collected. Analyses of samples was done from Oct 12, 2010, to Jan 10, 2011. Two of 50 water samples and 51 of 171 seepage samples from throughout the centre and surrounding areas of New Delhi were bla_{NDM-1} positive (figure 1); however, eight seepage samples did not give a positive result with PCR alone (figure 2). All 70 sewage effluent samples from Cardiff were negative for bla_{NDM-1}.

The two positive water samples were from designated public water supplies; one was taken west of the Yamuna River in the district of Ramesh Nagar and the other just south of the Red Fort. The NDM-1-positive seepage samples were collected from sites on both sides of the Yamuna River, including locations close to the main commercial and financial district around Connaught Place. Positive samples were also collected close to the Sir Ganga Ram Hospital and other public areas, including Gol Market.

All seepage samples grew bacteria (including Enterobacteriaceae) that were resistant to cefotaxime, and 166 of 171 grew bacteria on media containing 0·5 mg/L meropenem. All the concentrated water samples grew bacteria on media containing cefotaxime and 14 of 50 grew on media containing meropenem. bla_{NDM-1}-positive bacteria was recovered from 12 of 171 seepage samples and two of 50 water samples. Some samples contained multiple bla_{NDM-1}-positive species, although we cannot exclude the possibility that some organisms gained or lost the gene during the handling procedures.

20 NDM-1-positive strains were present in the samples (table 1), including the opportunistic Enterobacteriaceae *Citrobacter freundii*, *E coli*, and *K pneumoniae*, which commonly carry bla_{NDM-1} in hospitals in India and elsewhere,[4,8,15] as well as the pathogenic species *Shigella boydii* and *V cholerae*, and *Aeromonas caviae* (a probable cause of gastroenteritis). Non-fermentative Gram-negative bacteria not previously reported to carry bla_{NDM-1} were also present comprising *Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas pseudoalcaligenes, Pseudomonas oryzihabitans, Sutonella indologenes, Stenotrophomonas maltophilia, Achromobacter* spp, and *Kingella denitrificans*.

Detailed plasmid analysis, by methods previously described,[3,4] was done on isolates that were positive for bla_{NDM-1}. Passaging over 14 days in media with and without meropenem 0·5 mg/L showed that 12 of these 20 isolates, including all the Enterobacteriaceae, carried stable bla_{NDM-1} plasmids; however, many of the non-fermenters—isolates B-3-2, 1-19, 21-9, 72-28, W32-17, W38-14, W38-16, and W38-17—lost their plasmids within 48 h (two passages) with or without meropenem (0·5 mg/L).

Two isolates, *A caviae* (107-5) and *V cholerae* (116-14), carried bla_{NDM-1} on their chromosomes, although the *V cholerae* isolate also carried bla_{NDM-1} on a 400 kb plasmid (table 1). Plasmid profiling of the other isolates showed that they carried bla_{NDM-1} on plasmids ranging in size from 140 to 400 kb (table 1, figure 3). All the plasmids from Enterobacteriaceae isolates were the same size (140 kb). Where typeable, these plasmids, and a 170 kb plasmid from *V cholerae* 116-17, were IncA/C types, as identified by Southern blotting first to detect bla_{NDM-1} followed by use of a specific *rep/inc* probe with a method modified from that of Carattoli and colleagues.[16] Four of the 12 isolates that had stable plasmids possessed A/C replicon regions, whereas the remainder had non-typeable plasmids.

All of the Enterobacteriaceae isolates positive for bla_{NDM-1} had multiresistant phenotypes, including raised carbapenem minimum inhibitory concentrations (table 1), thus resembling NDM-1-positive bacteria from previous studies.[8,15,17] Typical NDM-1-type resistance was also seen in both *V cholerae* isolates and, aside from a low ceftazidime minimum inhibitory concentration, in *P putida* 107-7. Assessment was harder for the *A caviae* and the *S maltophilia* isolates, because these species and genera often have chromosomal carbapenemases distinct from NDM-1; nevertheless, these isolates also had broad resistance compatible with carriage and expression of bla_{NDM-1}. Despite being bla_{NDM-1}-positive, nine non-fermenters were less resistant than the Enterobacteriaceae (table 1) but were substantially more resistant than typical members of these species. Antibiogram profiling and molecular typing by pulsed-field gel electrophoresis showed that the two *V cholerae* isolates from site 116 were distinct.

Table 1 shows that bla_{NDM-1} plasmids vary in type and size, and occur in disparate bacteria. We therefore attempted to transfer bla_{NDM-1} from those bacteria that had stable plasmids into recipient species (*E coli*, *S enteritidis*, and *S sonnei*), and assessed the transfer frequency at temperatures relevant to the Indian environment. Isolates were classed as being unable to transfer bla_{NDM-1} into the recipient species if the frequency of transfer was 10^{-9} or lower. All the isolates with stable bla_{NDM-1}-encoding plasmids were able to transfer these to *E coli* J53 at 30°C and all except *S boydii* 65-5 did so at 25°C, whereas only *E coli* 3-1 achieved conjugative transfer at 37°C (table 2). Transfer to *S enteritidis* was less efficient, with seven of 12 capable of transfer at 30°C, only one (*K pneumoniae* 25-4) capable of transferring its plasmid at 25°C, and no transfers at 37°C. The transfer of bla_{NDM-1} plasmids into *S sonnei* was even less efficient, with only 117-4 (*C freundii*) and 25-4 (*K pneumoniae*) able to transfer at 30°C, and no transfers at 25°C or 37°C. Where transfer was achieved, frequencies at 30°C were one to 10 000 times higher than at 25°C and were one to 1 000 000 times higher than at 37°C; transfer into the *S enteritidis* and *S sonnei* recipients was ten to 1000 times less efficient than into *E coli* J53. *A caviae* 107-5 transferred bla_{NDM-1} into *E coli* J53 despite the fact that its bla_{NDM-1} was chromosomally encoded.

Discussion

NDM-1 β-lactamase is widely disseminated in New Delhi and has spread into key enteric pathogens. These data present an account of the environmental distribution of bacteria carrying the NDM-1 gene in New Delhi (panel). The two water sources that were bla_{NDM-1}-positive are used for drinking and food preparation as well as personal washing and cleaning of clothes. Whether these data can be extrapolated to other Indian cities is unknown, but clearly there is an urgent need for broad epidemiological and environmental studies to be done, not just in India, but also in Pakistan and Bangladesh, which are source countries for other exported cases of infection and colonisation with bla_{NDM-1}-positive bacteria.[18,19]

Some of the seepage samples failed to give a positive result by PCR alone, seemingly reflecting the increased sensitivity of DNA hybridisation. Also, although direct probing of PCR products showed good discrimination between negative and positive samples, some PCR products from seepage swabs appeared smeared, suggesting that DNA degradation by endonucleases occurred during handling. Such degradation probably occurred when the swabs were washed in 0·5 mL of molecular grade water before PCR, and suggests a high concentration of bacterial endonucleases in the samples. This inference is further supported by the finding that, when washed swabs were stored in a fridge overnight and the experiment repeated, 46 of 51 of the initially positive samples then appeared negative.

The sample volumes collected were very small and some bla_{NDM-1} plasmids were unstable. Consequently, the dissemination of bla_{NDM-1} is probably more extensive than indicated by the data presented here.

This study also emphasises the extent to which bla_{NDM-1} can disseminate among species, including not only enterobacteria but also aeromonads, vibrio, and various non-fermenters. These data reflect the gene's association with promiscuous plasmids—most of them IncA/C or non-typeable elements. This result is in keeping with previous data from clinical isolates, where bla_{NDM-1} was found in a wide range of species recovered from infected or colonised patients.[4,7] Despite possessing an intrinsic metallo-β-lactamase gene, bla_{L1}, *S maltophilia* 116-4 also carried a stable NDM-1-positive plasmid, suggesting that carbapenem resistance alone is not the sole selection criterion for maintaining bla_{NDM-1}-encoding plasmids.[20]

Some of the isolates—including all the Enterobacteriaceae, aeromonads, and *V cholerae* that were positive for bla_{NDM-1}—had the extreme drug-resistance profiles typical of clinical isolates with the NDM-1 enzyme,[4,8,15] along with stable, transferable expression of bla_{NDM-1}. Others, consisting of many of the non-fermenters, were little or no more resistant than typical members of their species, despite being bla_{NDM-1}-positive, as confirmed by sequencing. This finding could be because high-level resistance requires additional factors not present on these plasmids or because, owing to its promoter sequences, bla_{NDM-1} is not well expressed in some non-fermenters.

Two samples contained bla_{NDM-1}-positive strains of the classic pathogens *V cholerae* and *S boydii*. Although the primary treatment of cholera is based on rehydration,[21] antibiotics are used to reduce excretion of the bacteria, and any accumulation of resistance in the species must be viewed with concern. Antibiotics reduce the duration and severity of shigellosis,[22] but the genus shows increasing resistance to the typical treatments used—namely, ampicillin, co-trimoxazole, nalidixic acid, and ciprofloxacin.[23–25] Clinical isolates of *S boydii* from India are mostly susceptible to these antibiotics;[26] by contrast,

S boydii 65-5—the bla_{NDM-1} *Shigella* isolate from this study—was resistant to all these drugs as well as to oxyimino-cephalosporins, which are increasingly used as first-line therapy, and to azithromycin (data not shown), another treatment option.[26,27] Dysentery caused by strain 65-5 is potentially untreatable.[28]

NDM-1-positive Enterobacteriaceae carried similarly sized (140 kb) plasmids, corresponding to the size reported for bla_{NDM-1} plasmids by others.[4] These transferred to recipient *E coli*, *S enteritidis*, and *S sonnei* at higher frequencies at 30°C than at 25°C or, particularly, at 37°C; however, whether this finding is a result of species or strains difference is unclear, especially in view of the close relation between *E coli* and *Shigella* spp. This finding has serious implications for environmental transfer given that the average daily peak temperature in New Delhi reaches 30°C and a temperature of 30°C lies within the daily high–low range for the 7 months from April to October inclusive (figure 4). This period includes the monsoon season, when floods and drain overflows are most likely, which potentially disseminates resistant bacteria. In view of the lower transfer frequencies at 37°C than at 30°C, transfer in the environment might be more important than in the gut, even for those species of Enterobacteriaceae represented in the typical human flora. This aspect also highlights a deficiency with many published plasmid studies, which attempt in-vitro conjugation experiments only at 37°C.[29] However, thermosensitivity plays a part in the conjugative apparatus of plasmids of the IncHI families, and a key gene in the transfer locus is transcribed in a temperature-dependent manner, with substantially reduced levels of expression at 37°C compared with at 30°C.[30]

The results of this study suggest widespread environmental contamination and are in keeping with the facts that not all of the UK or European patients with bla_{NDM-1}-positve bacteria who had visited India were admitted to hospital there[4,11,31] and that many patients from Chennai who had bla_{NDM-1}-positive clinical isolates had no recent history of hospital admission.[4] Rather than being a purely nosocomial problem, bacteria with this resistance seem to be circulating in the community; thus they may be imported into hospitals with admitted patients and probably enriched within the gut flora when these patients, for whatever reason, receive antibiotic treatment or prophylaxis. The risk posed by the community circulation of multiresistant bacteria is emphasised by the recent study by the ReAct group,[11] which showed that seven of eight tourists returning to Sweden from India had colonisation by gut bacteria that produced extended-spectrum β-lactamases that they did not carry before departure, and is further supported by the high concentration of extended-spectrum β-lactamases in community-onset as well as hospital-onset intra-abdominal infections in India.[32] Similar colonisation studies on travellers and residents are urgently needed for NDM-1 carbapenemase.

Oral–faecal transmission of bacteria is a problem worldwide, but its potential risk varies with the standards of sanitation. In India, this transmission presents a serious problem, as highlighted by a recent UN report that showed that 650 million citizens do not have access to a flush toilet and even more probably do not have access to clean water.[33] The sewerage system in New Delhi is unable to cater for the city's population and only 60% of the population is served by the sewerage system, as emphasised by studies sponsored by Water Aid India.[12,34–37]

This study shows that international surveillance of resistance needs to be established. Such surveillance must incorporate environmental sampling as well as examination of clinical isolates and cover Pakistan and Bangladesh, because these are also source countries for exported cases. These are pressing needs if the ability to treat severe infections in vulnerable patients is to be maintained. The next step in this analysis should be to sample carriage in south Asia, both in residents and travellers. We would be delighted to advise and help in any capacity to take such studies forward in partnership with Indian, Pakistani, and Bangladeshi scientists, clinicians, and government officials.

Panel: Research in context

Systematic review

We searched PubMed, with no date limits set, for the terms "NDM-1", "surveillance", and "environmental". There are no controlled studies published examining the presence of NDM-1 in either water or waste matter. Furthermore, we extended the search to include "carbapenemases" and found three previous studies; however, these studies did not examine waste material or drinking water.

Interpretation

This is the first molecular study to examine for the presence of a carbapenemase gene and its corresponding plasmids in environmental bacteria. Our results clearly show the advantage of in-depth molecular studies when assessing antibiotic resistance and prove that environmental sampling in selected countries can be as important as clinical sampling.

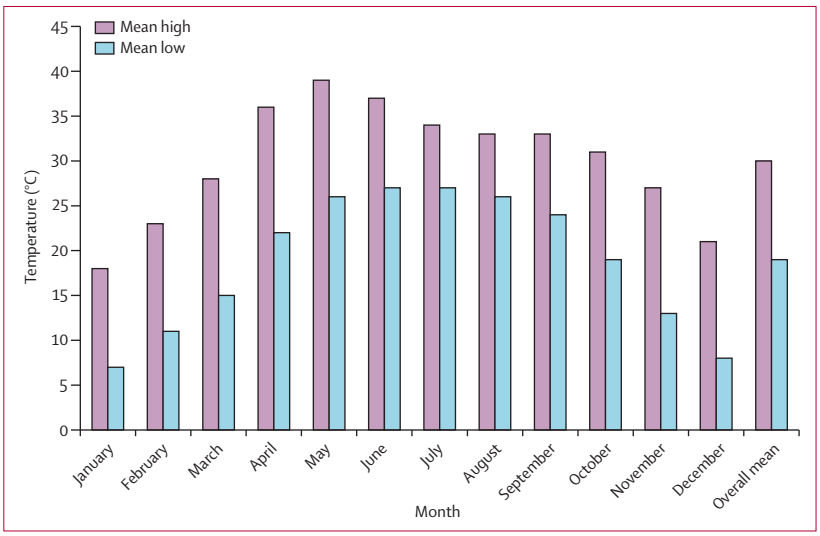

Figure 4: **Mean high and low temperatures for New Delhi**

Contributors

Authors from Cardiff University (TRW, JW, and MAT) undertook the laboratory work and wrote the manuscript. Channel 4 News collected the swabs and water samples and documented their location. DML interpreted the data and contributed to the editing of the manuscript.

Conflicts of interest

DML has received conference support from numerous pharmaceutical companies, and also holds shares in AstraZeneca, Merck, Pfizer, Dechra, and GlaxoSmithKline, and, as Enduring Attorney, manages further holdings in GlaxoSmithKline and Eco Animal Health. TRW has received payment for lectures from Pfizer and travel, accommodation, or meeting expenses from bioMérieux. All other authors declare that they have no conflicts of interest.

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