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Exploring the potential reservoirs of non specific TEM beta lactamase (bla_{TEM}) gene in the Indo-Gangetic region: A risk assessment approach to predict health hazards

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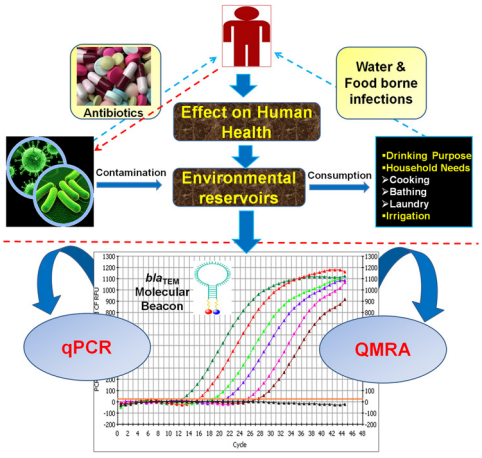
HIGHLIGHTS

- Prevalence of TEM-β-lactamase resistant bacteria in environmental regime.
- The utility of molecular beacon based qPCR in targeting bla_{TEM} gene.
- Assay useful in formulating risk assessment strategies to combat infections.
- Quantitative Microbial Risk assessment (QMRA) approach to predict human health risk.

GRAPHICAL ABSTRACT

Schematic representation of developed qPCR strategy in Molecular Beacon format to target bla_{TEM} gene encoding TEM-β-lactamase resistance in Indo-Gangetic region.



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ABSTRACT

The emergence of antimicrobial resistant bacteria is an important public health and environmental contamination issue. Antimicrobials of β-lactam group accounts for approximately two thirds, by weight, of all antimicrobials administered to humans due to high clinical efficacy and low toxicity. This study explores β-lactam resistance determinant gene (bla_{TEM}) as emerging contaminant in Indo-Gangetic region using qPCR in molecular beacon format. Quantitative Microbial Risk Assessment (QMRA) approach was adopted to predict risk to human health associated with consumption/exposure of surface water, potable water and street foods contaminated with bacteria having bla_{TEM} gene. It was observed that surface water and sediments of the river Ganga and Gomti showed high numbers of bla_{TEM} gene copies and varied significantly ($p < 0.05$) among the sampling locations. The potable water collected from drinking water facility and clinical settings exhibit significant number of bla_{TEM} gene copies (13 ± 0.44–10200 ± 316 gene copies/100 mL).

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It was observed that *E.crassipes* among aquatic flora encountered in both the rivers had high load of bla_{TEM} gene copies. The information on prevalence of environmental reservoirs of bla_{TEM} gene containing bacteria in Indo-Gangetic region and risk associated will be useful for formulating strategies to protect public from menace of clinical risks linked with antimicrobial resistant bacteria.

1. Introduction

The emergence of antimicrobial resistance has jeopardized the prevention and treatment/policy to combat fatal infections caused by pathogenic microbes [1]. Anthropogenic as well as naturally occurring antimicrobial resistance has been observed in microorganisms [2]. It has been suggested that persistence of natural antimicrobials in the environment provided long-term selective pressure for the emergence and transmission of resistance-conferring genes in naive organisms [2,3]. The antimicrobial resistance determinant genes originated in the microorganisms are transferred to currently observed pathogenic bacteria in clinical settings by horizontal gene transfer through mobile genetic elements [4–6]. Humans may acquire infections from antimicrobial resistant microorganisms irrespective of their origin. Therefore, the occurrence of antimicrobial resistant bacteria (ARB) and antimicrobial resistance determinant genes (ARGs) became an important public health and environmental contamination issue [7]. This is perhaps due to the transference of ARB and ARGs across international boundaries by human travelers, animal and insect vectors, agricultural products and surface water, after their initial selection and local dissemination [8].

Antimicrobials classified into β-lactam group (penicillins, cephalosporins and carbapenems) accounts for approximately two thirds, by weight, of all antibiotics administered to humans due to high clinical efficacy and low toxicity [9]. Bacterial pathogens confer resistance to β-lactam antimicrobials by production of β-lactamases which break down β-lactams by hydrolyzing four – membered β-lactam ring [10]. TEM–β-lactamases encoded by bla_{TEM} gene represents one of the most clinically significant families of β-lactamases. The family of TEM–β-lactamases include both broad spectrum and extended – spectrum β-lactamases and are most prevalent and widely distributed plasmid mediated enzymes [5,11].

'Environmental reservoirs' are defined as locations out of the human body within the niche favouring bacterial persistence and replication in the environment, and pathogen transmission to susceptible hosts [12]. The environmental reservoirs, on occasion, can act as vectors, i.e. an organism or substrate that transmits a pathogen to the final host [12]. The occurrence of pathogenic ARG (including bacteria resistant to β-lactamase group) in environmental reservoirs (terrestrial soils, aquatic sediments or beach sand, aquatic vegetation) has been demonstrated in Indo-Gangetic region [13–16]. Therefore, it is important to increase our understanding of the dynamics of this group in the environment. Hence, in this study, environmental reservoirs of β lactam resistant bacteria were identified by quantifying TEM–β-lactamase gene in an urban stretch of Gangetic riverines, potable water and street food in the Indo Gangetic region.

2. Materials and methods

2.1. Designing of primers and probes

In this study for the specific quantification of bla_{TEM} gene in environmental samples, a universal molecular beacon (MB) (5′- FAM: CACGCCTGCGGCCAACTTACTTCT GACAACGGCGTG-DABCYL −3′) and its corresponding primers (bla_{TEM} F': 5′- TGCCATAACCATGAGTGATAAC-3′ and bla_{TEM} R': 5′-CAAGGCGAGTTACATGATCC-3′) were designed against highly conserved sequences (466 bp) of bla_{TEM} gene using Beacon Designer 8.0: Premier Biosoft International (Tables S1 &S2 in supplementary materials). Probe and primers were synthesized from Metabion (Gmbh, Germany).

2.2. Culture-free quantification of bla$_{TEM}$ gene copies in potential environmental reservoirs

To identify potential environmental reservoirs of β lactamase resistance, two Indian perennial rivers (Ganga River, Kanpur city and the river Gomti, Lucknow city) flowing through northern India, water from a potable water distribution network at Lucknow city, water from drinking water facility at major hospitals located in Lucknow city and street food samples were selected to quantify the bla_{TEM} gene copies (GC) through culture-free MB based qPCR assay. Replicate (n = 5) grab surface water samples (1 L) were collected and processed [17,18] in sterilized bottles from six sites (Site #1, Bithoor upstream; Site # 2, Ganga barrage; Site# 3, Bhairoghat (cremation ghat); Site# 4, Shuklaganj; Site# 5, Nanaraoghat; Site# 6, Jajmau) in the Ganga River covering a 30 km stretch (Fig. S2 in supplementary materials) and from eight sites (Site#1 Ghaila Bridge, Site#2 Gaughat, Site#3 Pakkapul, Site#4 Saheed Smarak, Site#5 Bhaisakund, Site#6 Piperaghat, Site#7 Chandiamau, Site#8 Indira jal Setu) in the river Gomti covering 10 km stretch (Fig. S3 in supplementary materials). Surface sediments (0–5 cm, ∼250 g) and aquatic plants/algae were also collected at selected sampling locations (n = 5) from each river bed (∼1 m distance) and processed [14,18,19]. Potable water samples (5 L) from each site in triplicate were collected from six sites (Site #1, Aishbagh water works; Site #2: Rakabganj; Site # 3: Hussainganj; Site #4: Kaiserbagh; Site # 5 Nakkhas and Site #6: Shastrinagar) located in potable water distribution network at Lucknow city (Fig. S4 in supplementary materials). Further, six hospitals, located in Lucknow city (Fig. 1), discharging their clinical waste water into urban sewerage were also selected to collect potable waters (5 L from each site in triplicate) for the present study and processed according to Singh et al. [20].

Fruit juices (2 L each in sterilized bottles), Street foods (25 g each except eggs) and garnishing materials (25 g each) were collected from six distinct localities (Site # 1, Aliganj; Site # 2, Indira nagar; site #3, Gomtinagar; site # 4, Sadar Bazar; site # 5, Rajajeepurum; site #6, Alambagh in triplicate (Fig. 2) and processed according to Singh et al. [14]. Multigenomic DNA was isolated from environmental samples (plant/water/sediment/street foods) through boiling prep as described by Jyoti et al. [19]. Quantitative enumeration of bla_{TEM} GC in potential environmental reservoirs was carried out by using a standard curve generated from serially ten-fold diluted (10^8–1 GC) plasmid DNA stock in sterile Milli-Q water. Three replicates of each dilution were added to each qPCR reaction.

2.3. Risk assessment studies

Health risk associated with exposure to bacteria exhibiting bla_{TEM} gene in surface water, potable water, street foods and fruit

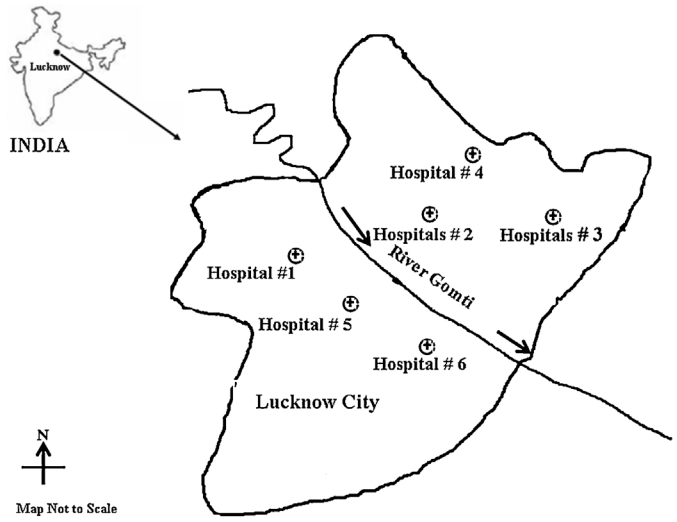

Fig. 1. Diagrammatic representation of sampling sites selected for collection of potable water from drinking water facility in clinical establishments.

juices was assessed by Quantitative microbial risk assessment (QMRA) approach, with an assumption that only one gene copy was present per cell of bacteria. According to Haas et al. [21], QMRA involves a sequence of interrelated steps: a) hazard identification; b) exposure assessment; c) dose-response assessment; d) risk characterization. The different steps are presented as follows:

2.3.1. Hazard identification

In this study, *bla*$_{TEM}$ gene copies were chosen as the main hazard for the risk assessment for exposed populations, either through exposure or consumption of environmental samples.

2.3.2. Exposure assessment

Exposure assessment involves the determination of the "amount or number of organisms that occurs as a dose" during a single event exposure or the total number of *bla*$_{TEM}$ gene copies that will constitute a set of multiple [21]. In this study, single event exposure was considered and four modes were assessed: (a) Ingestion of surface water contaminated with bacteria exhibiting *bla*$_{TEM}$ gene

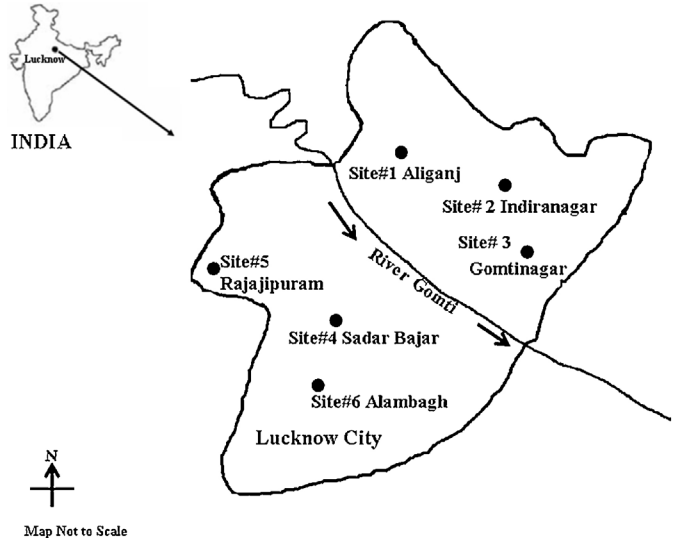

Fig. 2. Diagrammatic representation of sampling sites selected for collection of street food in a highly populated north Indian city located in Indo-Gangetic region for quantitative enumeration of *bla*$_{TEM}$ gene copies.

(b) Ingestion of potable water contaminated with bacteria exhibiting *bla*$_{TEM}$ gene (c) Consumption of fruit juices and (d) Consumption of street foods. The copies of *bla*$_{TEM}$ gene in each exposure route was used to determine risk for best and worst case scenarios. The lowest gene copies in an exposure medium was considered as a best case scenario whiles the highest number of gene copies recorded in the same medium was used to determine the worst case scenario.

2.3.3. Dose-response assessment

Dose response assessment was undertaken to assess the relationship between the dose of *bla*$_{TEM}$ copies ingested by exposed populations and the probability of infection. In this study, the β-Poisson dose response model, developed by Navarro et al. [22] for assessing *E. coli* infection, was used. The probability of infection was therefore calculated based on the common beta-poison model as shown in Eq. (1):

$$p(d) = 1 - \left(1 + \left(\frac{d}{N_{50}}\right)\left(2^{\frac{1}{\alpha}} - 1\right)\right)^{-\alpha} \quad (1)$$

Where P(d) = Probability of Infection, d = dose of *bla*$_{TEM}$, N_{50} = Median Infection dose and α = Dimension less Infectivity Constant

2.3.4. Risk characterization

In the risk characterization all the outcomes of the hazard identification, exposure assessment and dose response assessment were combined to characterize the *bla*$_{TEM}$ infection for the exposed population. The risk characterization was done separately for the exposure scenarios as detailed under the exposure assessment.

2.4. Statistical analyses

For comparison of PCR amplification efficiencies and detection sensitivities among different experiments, slopes of the standard curves were calculated by performing a correlation and regression analysis through iCycle iQ^{TM} Real-Time Detection System Software Version 3.0A. Amplification efficiency (E) was calculated as shown in Eq. (2):

$$E = (10^{-1/\text{slope}})^{-1} \quad (2)$$

The comparison of contamination level of six sites in river Ganga and eight sites in its tributary river Gomti, in terms of *bla*$_{TEM}$ GC in surface water and sediment was performed using one way analysis of variance [23]. Duncan's Multiple Range test (DMRT) was used to compare the means [23]. Similar approach was adopted for all environmental samples.

3. Results and discussion

3.1. Sensitivity and specificity of the molecular beacon based qPCR assay targeting bla$_{TEM}$ *gene*

All the reference strains used in this study exhibit *bla*$_{TEM}$ gene except *Escherichia coli* ATCC 25922 (Table S3 in supplementary materials). Eighty eight environmental isolates (Enterohemorrhagic *Escherichia coli*: 41, Enterotoxigenic *Escherichia coli:* 23, *Enterococci* spp.: 12, *Salmonella* spp.: 12) retrieved from surface/potable water have been screened for the presence of *bla*$_{TEM}$ gene (Table S3 in supplementary materials). It was evident from the results that designed assay was capable of quantifying *bla*$_{TEM}$ gene copies in genetically diverse pathogenic bacteria (Table S4 in supplementary materials) and this detailed comprehensive monitoring strategy facilitates detection across the species irrespective of variations in the gene. The assay designed in this study could detect lowest 10 genomic copies of *bla*$_{TEM}$ gene (PCR efficiency:

Table 1
Quantitative enumeration of bacterial pathogens exhibiting bla_{TEM} gene copies (GC) in Surface Water and Sediments of two Sewage-Impacted River.

Sampling sites[§]	Surface water[*,†,‡] (bla_{TEM} GC/100 mL)		Sediments[*,†,‡] (bla_{TEM} GC/100 g)	
	Ganga	Gomti	Ganga	Gomti
Site # 1	$^{e}4.3 \times 10^{3} \pm 1.1 \times 10^{2}$	$^{e}8.1 \times 10^{3} \pm 2 \times 10^{2}$	$^{e}5.3 \times 10^{4} \pm 5.9 \times 10^{2}$	$^{e}2.9 \times 10^{4} \pm 9.2 \times 10^{2}$
Site # 2	$^{f}2.3 \times 10^{2} \pm 7 \times 10^{1}$	$^{c}6.6 \times 10^{5} \pm 2 \times 10^{2}$	$^{f}5.6 \times 10^{3} \pm 6.5 \times 10^{2}$	$^{c}2.2 \times 10^{6} \pm 5.0 \times 10^{3}$
Site # 3	$^{d}2.4 \times 10^{4} \pm 4.4 \times 10^{3}$	$^{d}1.4 \times 10^{4} \pm 6 \times 10^{3}$	$^{d}8.9 \times 10^{5} \pm 2.3 \times 10^{2}$	$^{d}7.1 \times 10^{5} \pm 2.1 \times 10^{3}$
Site # 4	$^{b}4.9 \times 10^{6} \pm 5 \times 10^{5}$	$^{b}4.1 \times 10^{6} \pm 1 \times 10^{3}$	$^{b}7.9 \times 10^{7} \pm 1.7 \times 10^{5}$	$^{b}9.1 \times 10^{8} \pm 3.7 \times 10^{4}$
Site # 5	$^{c}1.5 \times 10^{5} \pm 4 \times 10^{2}$	$^{a}9.3 \times 10^{7} \pm 3.5 \times 10^{3}$	$^{c}3.7 \times 10^{6} \pm 8.4 \times 10^{5}$	$^{a}1.4 \times 10^{9} \pm 4 \times 10^{6}$
Site # 6	$^{a}2.10 \times 10^{7} \pm 5 \times 10^{5}$	$^{d}4.9 \times 10^{4} \pm 1 \times 10^{2}$	$^{a}9.9 \times 10^{9} \pm 1.9 \times 10^{5}$	$^{d}8.3 \times 10^{5} \pm 3.3 \times 10^{3}$
Site # 7		$^{e}8.9 \times 10^{3} \pm 3.6 \times 10^{1}$		$^{e}3.1 \times 10^{4} \pm 9.6 \times 10^{2}$
Site # 8		$^{e}8.9 \times 10^{3} \pm 3.1 \times 10^{1}$		$^{e}2.9 \times 10^{4} \pm 6.1 \times 10^{2}$

Identical superscripts on means denote insignificant ($p > 0.05$) difference between the means according to Duncan's Multiple Range Test.
[*]Values are mean ($n = 5$) ± SD; $Y = -3.327X + 34.179$, correlation coefficient $r = 0.997$, PCR efficiency: 99.8%.
[§]Sampling sites: River Gomti at Lucknow city: Site #1 Ghaila Bridge; Site #2 Gaughat, Site #3 Pakkapul, Site #4 Saheed Smarak, Site #5 Bhaisakund, Site #6 Piperaghat, Site #7Chandiamau, Site #8 Indira jal Setu; River Ganga at Kanpur: Site #1 Bithoor, Site #2 Ganga barrage, Site #3 Bhairaoghat, Site #4 Shuklaganj, Site #5 Nanaraoghat, Site #6 Jajmau.
[†]No amplification was observed when 5 μL DNA containing 10^4 genomic equivalents of *Escherichia coli* ATCC 25922 lacking bla_{TEM} gene used as negative control or 5 μL sterile Milli-Q® (Millipore, Billerica, MA, USA) water used as no template control in qPCR assay designed to quantify bla_{TEM}.
[‡]Column wise one way ANOVA in complete randomized block design significant at 5% level.

99.6%; $Y = -3.332X + 41.234$, correlation coefficient $r = 0.996$). However, the presence of background DNA from *E. coli* ATCC 25922 lacking bla_{TEM} gene (10^6 genomic copies/PCR) dropped the sensitivity of the assay 1000-fold (Table S5 in supplementary materials). It has been reported earlier that the presence of nonspecific DNA reduce the detection limit of the qPCR [24,25]. Further, this comprehensive monitoring strategy followed by risk assessment is likely to lead to more effective control of the antibiotic resistance epidemics.

3.2. Bacteriological quality and quantification of bla$_{TEM}$ gene copies in reverine environment of two perennial rivers flowing in Indo-Gangetic region

Surface water and sediments collected from all the sampling sites in the river Ganga and river Gomti exhibit significantly (one way ANOVA, $p < 0.05$) high numbers of the total coliforms, fecal coliforms and bla_{TEM} gene copies (Table 1, Table S6 in supplementary materials). However, the number of bla_{TEM} gene copies varied significantly (DMRT $p < 0.05$) among the sampling locations 2, 4 and 5 (Table 1). Sites # 1, 7 and 8 in the river Gomti exhibit no significant difference between bla_{TEM} gene copies present in surface water (DMRT, $p > 0.05$) or sediments (DMRT, $p > 0.05$). Similarly, no significant difference was observed in bla_{TEM} gene copies retrieved from surface water or sediment of sites #3 and #6 of the river Gomti. High numbers of bla_{TEM} gene copies (one way ANOVA, $p < 0.05$) were found in surface water and sediments of the river Ganga. The number of bla_{TEM} gene copies in the river Ganga water or sediment varied significantly (DMRT, $p < 0.05$) at different sampling locations.

The river Ganga (traveling 2510 km across the Indian subcontinent) and its tributaries, the major source of water for drinking and irrigation (40% of the total water requirement) in the Gangetic planes of northern India are overburdened due to urbanization and exponential population growth. In this study, total coliforms and fecal coliforms recorded at all the sampling stations in the rivers Ganga and Gomti exceeded the standards set by regulatory authorities for surface water reservoirs to be used for drinking and recreational purposes [26,27]. Further, the sediments of both the rivers served as reservoirs of the total coliform and fecal coliform bacteria. It has been reported that the river Ganga and the river Gomti receive domestic sewage, waste water from poultry, dairy farms, clinical settings, burnt and half burnt animal and human carcasses during their course in the selected study stretch [19,20]. It has been documented that increased concentrations of fecal indicator bacteria in sediments and aquatic vegetation have prompted concern because environmental reservoirs of fecal indicator bacteria may disrupt the correlation between indicator organisms, pathogens, and human health risks [28].

3.2.1. Aquatic flora as potential environmental reservoir of β-lactamase resistance in Indo-Gangetic region

The composition of aquatic flora varied among different sites in both the rivers. In this study, *E. crassipes* (sites # 2–5) was the dominant species in the river Gomti followed by *P. crispus* (sites # 1–3) and *P. pectinatus* (sites # 2–3). *R. esculentus* and *N. indica* were present only at site #8 (the Gomti river) while *Spirogyra* spp. and *P. glabrum* were encountered only at site #7 of the same river (Table 3). *P. crispus* was collected from all the sites selected in the river Ganga except site #4. Further, *E. crassipes* and *P. pectinatus* were absent at sites #1 and 4 of the river Ganga. *P. glabrum* and *R. esculentus* were present only at site # 6 in the river Ganga (Table 2). *Spirogyra* spp. and *N. indica* were collected from site#3 and site# 5 in the river Ganga, respectively (Table 2).

It has been observed that aquatic flora encountered in both the rivers exhibit high load of the total coliform and fecal coliform bacteria (Table 2). The *E. crassipes* plants collected from both the rivers exhibit maximum bla_{TEM} GC. A significant variation in bla_{TEM} GC was observed between *E. crassipes* samples from different sites (DMRT, $p < 0.05$). Other plant species comprising flora of these two rivers also harbor significant number of bla_{TEM} GC in comparison to samples of these plants from pristine sites (Table 2).

The Indo- Gangetic region, a large and fertile plain encompassing most of northern and Eastern India, Pakistan, Nepal and entire Bangladesh has been demonstrated to exhibit pathogenic bacteria harboring resistance to β-lactamases in aquatic ecosystems, potable water distribution system and street food [17,29,30]. The prevalence of bla_{TEM} gene in surface water, sediment and aquatic flora of the two perennial rivers of Indo-Gangetic region indicates that aquatic plants including algae may serve as reservoirs of β lactamase resistance in natural water resources. The possibility of long-term persistence of bla_{TEM} gene copies harboring bacteria in aquatic flora also has important ecological and public health implications. Environmental reservoirs of TEM-β-lactamase resistance encoding gene could disseminate TEM-β-lactamase resistance across large geographical boundaries by human travelers, animal and insect vectors, agricultural products, and surface water.

The previous studies demonstrated occurrence of β-lactamase resistance encoding genes in Chinese rivers [31]. Further, occurrence of β-lactamase resistance bacteria in rivers of Australia (Brisbane River and its tributaries), Germany (Rhine and Danube and their tributaries) and Spain [32,33] confirms that the bla_{TEM} gene copies obtained in this study in different environmental samples were in accordance to other reported studies. The high-

Table 2
Bacterial density and prevalence of β-lactamase resistance in terms of bla_{TEM} gene copies in aquatic flora in sewage-impacted rivers of Indo-Gangetic region.

Aquatic Flora	Total coliforms (MPN/100 g)*	Fecal coliforms (MPN/100 g)*	Gene copies /100 g*,†
River Gomti§			
P. Crispus¶			
Site#1	$^{c}1.8 \times 10^{4} \pm 4.3 \times 10^{2}$	$^{b}6.8 \times 10^{3} \pm 2.3 \times 10^{2}$	$540^{b} \pm 18$
Site#2	$^{a}9.2 \times 10^{7} \pm 3.1 \times 10^{6}$	$^{a}7.6 \times 10^{4} \pm 2.3 \times 10^{3}$	$490^{c} \pm 15$
Site#3	$^{b}1.3 \times 10^{5} \pm 3.2 \times 10^{4}$	$^{b}4.3 \times 10^{3} \pm 2.2 \times 10^{2}$	$780^{a} \pm 32$
P. pectinatus¶			
Site#1	$8.3 \times 10^{4} \pm 3.3 \times 10^{3}$	$2.9 \times 10^{3} \pm 8.4 \times 10^{2}$	210 ± 11
Site#3	$9.8 \times 10^{6} \pm 4.7 \times 10^{5}$	$7.1 \times 10^{4} \pm 2.8 \times 10^{3}$	340 ± 14
E. crassipes¶			
Site#2	$^{c}1.8 \times 10^{4} \pm 5.04 \times 10^{3}$	$^{c}6.3 \times 10^{3} \pm 1.51 \times 10^{2}$	$600^{c} \pm 3$
Site#3	$^{b}5.3 \times 10^{5} \pm 1.87 \times 10^{4}$	$^{b}4.3 \times 10^{4} \pm 2.36 \times 10^{3}$	$788^{b} \pm 3$
Site#4	$^{c}1.8 \times 10^{4} \pm 4.45 \times 10^{3}$	$^{c}7.3 \times 10^{3} \pm 1.98 \times 10^{1}$	$567^{d} \pm 3$
Site#5	$^{a}1.9 \times 10^{7} \pm 6.24 \times 10^{3}$	$^{a}6.4 \times 10^{5} \pm 1.34 \times 10^{3}$	$950^{a} \pm 2$
R. esculentus¶			
Site#8	$9.8 \times 10^{4} \pm 2.5 \times 10^{3}$	$8.1 \times 10^{3} \pm 2.9 \times 10^{2}$	400 ± 17
N. indica			
Site#8	$7.2 \times 10^{6} \pm 2 \times 10^{3}$	$1.2 \times 10^{5} \pm 4 \times 10^{3}$	156 ± 5
Spirogyra spp¶			
Site#7	$8.2 \times 10^{6} \pm 4.9 \times 10^{3}$	$9.2 \times 10^{4} \pm 2.7 \times 10^{3}$	870 ± 38
P. glabrum¶			
Site#7	$6.2 \times 10^{6} \pm 3.12 \times 10^{3}$	$7.5 \times 10^{5} \pm 2.7 \times 10^{4}$	670 ± 27
River Ganga‡			
P. crispus¶			
Site # 1	$^{d}1.3 \times 10^{6} \pm 4.2 \times 10^{4}$	$^{d}8.9 \times 10^{4} \pm 2.2 \times 10^{3}$	$780^{e} \pm 27$
Site # 2	$^{e}5.6 \times 10^{4} \pm 2.5 \times 10^{3}$	$^{e}7.3 \times 10^{3} \pm 3.6 \times 10^{2}$	$890^{d} \pm 23$
Site # 3	$^{c}9.4 \times 10^{7} \pm 2.2 \times 10^{6}$	$^{c}9.7 \times 10^{5} \pm 3.8 \times 10^{3}$	$1340^{c} \pm 60$
Site # 5	$^{b}5.5 \times 10^{8} \pm 2.8 \times 10^{6}$	$^{b}3.1 \times 10^{6} \pm 1.5 \times 10^{5}$	$1590^{b} \pm 64$
Site # 6	$^{a}3.8 \times 10^{9} \pm 4.7 \times 10^{7}$	$^{a}4.2 \times 10^{7} \pm 1.1 \times 10^{5}$	$1730^{a} \pm 88$
P. pectinatus¶			
Site # 2	$^{d}9.1 \times 10^{4} \pm 4.3 \times 10^{3}$	$^{d}3.6 \times 10^{3} \pm 5\ 0.6 \times 10^{2}$	$520^{c} \pm 21$
Site # 3	$^{c}5.6 \times 10^{5} \pm 1.1 \times 10^{4}$	$^{c}2.1 \times 10^{4} \pm 6.4 \times 10^{3}$	$432^{d} \pm 16$
Site # 4	$^{b}5.4 \times 10^{6} \pm 1.1 \times 10^{5}$	$^{b}2.1 \times 10^{5} \pm 6.4 \times 10^{4}$	$735^{b} \pm 30$
Site # 6	$^{a}3.5 \times 10^{7} \pm 1.8 \times 10^{6}$	$^{a}4.7 \times 10^{6} \pm 2.1 \times 10^{5}$	$990^{a} \pm 44$
E. crassipes¶			
Site#2	$^{b}1.9 \times 10^{5} \pm 6.76 \times 10^{3}$	$^{b}1.1 \times 10^{4} \pm 5.1 \times 10^{2}$	$1150^{c} \pm 46$
Site#3	$^{c}1.8 \times 10^{4} \pm 3.8 \times 10^{3}$	$^{c}6.3 \times 10^{3} \pm 2.16 \times 10^{2}$	$1550^{b} \pm 70$
Site#4	$^{b}2.1 \times 10^{5} \pm 6.3 \times 10^{3}$	$^{b}2.3 \times 10^{4} \pm 1.1 \times 10^{3}$	$847^{d} \pm 36$
Site # 6	$^{a}1.8 \times 10^{6} \pm 2.1 \times 10^{4}$	$^{a}8.3 \times 10^{5} \pm 2.\ 9 \times 10^{4}$	$1896^{a} \pm 85$
R. esculentus¶			
Site #6	$4.8 \times 10^{5} \pm 1.64 \times 10^{4}$	$9.8 \times 10^{4} \pm 3.4 \times 10^{3}$	360 ± 15
N. indica¶			
Site# 5	$3.8 \times 10^{4} \pm 1.2 \times 10^{3}$	$9.5 \times 10^{3} \pm 3 \times 10^{2}$	134 ± 4
Spirogyra spp.¶			
Site# 3	$7.6 \times 10^{5} \pm 1.7 \times 10^{4}$	$9.2 \times 10^{4} \pm 2.4 \times 10^{3}$	1699 ± 77
P. glabrum¶			
Site # 6	$7.2 \times 10^{6} \pm 2.52 \times 10^{3}$	$7.2 \times 10^{4} \pm 1.1 \times 10^{3}$	980 ± 44

Identical superscripts on means denote insignificant ($p > 0.05$) difference between the means according to Duncan' Multiple Range Test.
*Values are mean ($n = 5$) ± SD; $Y = -3.335X + 39.179$, correlation coefficient $r = 0.989$, PCR efficiency: 99.5%.
§The river Gomti at Lucknow: Site #1 Ghaila Bridge, Site #2 Gaughat, Site #3 Pakkapul, Site #4 Saheed Smarak, Site #5 Bhaisakund, Site #6 Piperaghat, Site #7Chandiamau, Site #8 Indira jal Setu.
‡The river Ganga at Kanpur: Site #1 Bithoor, Site #2, Ganga barrage;,Site #3 Bhairaoghat, Site #4 Shuklaganj, Site #5 Nanaraoghat, Site #6 Jajmau.
†No amplification was observed when 5 μL DNA containing 10^4 genomic equivalents of *Escherichia coli* ATCC 25922 lacking bla_{TEM} gene used as negative control or 5 μL sterile Milli-Q® (Millipore, Billerica, MA, USA) water used as no template control in qPCR assay designed to quantify bla_{TEM}.
¶Multigenomic DNA from each Plant species (listed in table) collected from pristine site was negative for target the gene. If one plant species occur more than two sites in one river, one way ANOVA followed by Duncan's Multiple Range test was used to find out significant variation in bla_{TEM} gene copies between the sites. One way ANOVA in complete randomized block design significant (*P. Crispus* and *E.crassipes* collected from the river Gomti; *P. Crispus P. pectinatus and E.crassipes* collected from the river Ganga) at 5% level.

est concentrations of *bla* genes in the Pearl and Haihe rivers in China were reported in range of $4.70 \pm 0.6 \times 10^3$ copies/mL and $3.0 \pm 1.0 \times 10^3$ copies/mL, respectively ($p > 0.05$) [31]. The concentration of bla_{TEM} genes in influent and effluent samples collected from wastewater treatment plants in Massachusetts was found to be 1.4×10^6 and 4.1×10^5 respectively [11].

The highest bla_{TEM} gene copies reported in the present study, in case of surface water, was $2.10 \times 10^7 \pm 5 \times 10^5$ for river Ganga and $9.3 \times 10^7 \pm 3.5 \times 10^3$ for the river Gomti. This further supports our results showing bla_{TEM} gene concentration in accordance to the globally published reports in sewage impacted Indian rivers.

Table 3
Determination of bla_{TEM} gene copies in potable water collected from potable water distribution system and drinking water facility at clinical settings in Lucknow city.

Potable water distribution System	bla_{TEM} GC/100 mL[†,§,*]	Clinical settings[¶]	bla_{TEM} GC/100 mL[†,§,*]
Site# 1 Aishbagh WW	ND[‡]	Hospital #1	$13^{c} \pm 0.44$
Site# 2 Rakabganj	$850^{d} \pm 25$	Hospital #2	$17^{b} \pm 0.48$
Site#3Hussainganj	$300^{e} \pm 9$	Hospital #3	$17^{b} \pm 0.66$
Site# 4 Kaiserbagh	$2700^{a} \pm 105$	Hospital #4	$14^{bc} \pm 0.27$
Site#5 Nakkhas	$995^{c} \pm 26$	Hospital #5	$25^{b} \pm 0.70$
Site#6 Shastrinagar	$1185^{b} \pm 50$	Hospital #6	$10200^{a} \pm 316$

¶Clinical settings at Lucknow city: Site #1: Hospital 1, Site # 2: Hospital 2, Site # 3: Hospital 3, Site # 4: Hospital 4, Site # 5:Hospital 5, Site # 6: Hospital 6.
*Values are mean (n = 5) ± SD; Y = −3.328X + 34.052, correlation coefficient r = 0.997, PCR efficiency: 99.8%.
‡ND: not detected.
†No amplification was observed when 5 μL DNA containing 10^4 genomic equivalents of *Escherichia coli* ATCC 25922 lacking bla_{TEM} gene used as negative control or 5 μL sterile Milli-Q® (Millipore, Billerica, MA, USA) water used as no template control in qPCR assay designed to quantify bla_{TEM}.
§Column wise one way ANOVA in complete randomized block design significant at 5% level. Identical superscripts on means denote insignificant ($p > 0.05$) difference between the means according to Duncan' Multiple Range Test.

3.3. Determination of bla$_{TEM}$ gene copies in potable water collected from water distribution system and clinical settings

It has been observed that the surface water from the river Gomti is processed by alum treatment, filtration and chlorination at Aishbagh Water works and distributed to the urban population of densely populated Lucknow city located in the Indo-Gangetic plain. The bla_{TEM} gene copies in potable water collected from water distribution network to an urban population ranged between 300 ± 9–2700 ± 105 GC/100 mL (Table 3). The level of bla_{TEM} gene copies in potable water varied significantly (DMRT, $p < 0.05$) at distinctly located sampling sites (Table 3). The potable water sample collected prior to distribution at Aishbagh Water works was negative for bla_{TEM} gene (Table 3).

Further, potable water collected from drinking water facility at clinical settings (Table 3) discharging their effluent in the river Gomti at Lucknow city exhibit significant (one way ANOVA, $p < 0.05$) number of bla_{TEM} gene copies (13 ± 0.44–10,200 ± 316 GC/100 mL).

Hospital waste is an important source of antimicrobial resistant bacteria into domestic sewage merging untreated into rivers of India and other countries [34,35]. During this study, bla_{TEM} gene copies were present in water samples collected from the drinking water facility of clinical settings discharging their waste water in to the sewage drains destined to merge in the river Gomti at Lucknow city. The presence of TEM-β-lactamase resistance in hospital environment especially in drinking water is again a critical issue related to recovery of immunocompromised patients from illness. Rusted pipelines of water distribution system running parallel to sewage collection lines result into contamination of potable water by β-lactamase resistant bacteria [18]. Prevalence of β-lactamase resistant bacteria and bla_{TEM} and bla_{SHV} copies in drinking water treatment and distribution systems of Michigan, Ohio has been reported and authors have suggested that the water distribution systems may serve as an important reservoir for the spread of antimicrobial resistance to opportunistic pathogens [36].

3.4. Street food articles as potential environmental reservoir of β-lactamase resistance

In this study, six juices and six food articles collected from distinct localities in a major city of Indo-Gangetic plain revealed that all the samples exhibit bla_{TEM} gene copies except mausammi juice at site # 1, 5–6 (Table 4). It has been noticed that potable water from urban water distribution network is used by vendors during processing of these food articles.

In this study, food/vegetables processed or irrigated by water exhibiting antimicrobial resistant bacteria emerged as potential

Table 4
Quantitative Enumeration of bla_{TEM} gene copies in Street food collected from different locations in the Lucknow City.

Sites[¶]	bla_{TEM} gene copies in fruit juices (bla_{TEM} GC/100 mL)[*,†,§]					
	Pineapple	Mausammi	Sugarcane	Orange	Pomegranate	Watermelon
Site # 1	$500^{d} \pm 20$	ND[‡]	$2630^{a} \pm 92$	$1080^{a} \pm 47$	$810^{a} \pm 22$	$750^{a} \pm 32$
Site # 2	$900^{a} \pm 42$	$105^{b} \pm 5$	$1080^{e} \pm 26$	$900^{c} \pm 42$	$628^{b} \pm 27$	$320^{d} \pm 12$
Site # 3	$670^{c} \pm 25$	$50^{c} \pm 3$	$1550^{d} \pm 55$	$720^{e} \pm 33$	$205^{e} \pm 8$	$570^{b} \pm 20$
Site # 4	$805^{b} \pm 24$	$150^{a} \pm 12$	$2140^{c} \pm 70$	$1005^{b} \pm 45$	$400^{d} \pm 15$	$430^{c} \pm 18$
Site # 5	$452^{e} \pm 20$	ND[‡]	$2362^{b} \pm 80$	$885^{d} \pm 27$	$518^{c} \pm 20$	$205^{e} \pm 6$
Site # 6	$252^{e} \pm 10$	ND[‡]	$845^{f} \pm 30$	$540^{f} \pm 25$	$85^{f} \pm 3$	$120^{f} \pm 4$

Sampling sites[¶]	bla_{TEM} gene copies in street food and its garnishing material (bla_{TEM} GC/100 g)[*,†,§]					
	Egg	Chowmein	Panipuri water	Raw Mutton	Coriander leaves	Mint leaves
Site # 1	$1045^{c} \pm 35$	$1920^{b} \pm 85$	$789^{c} \pm 21$	$2856^{b} \pm 97$	$1089^{a} \pm 23$	$889^{a} \pm 28$
Site # 2	$710^{e} \pm 30$	$1390^{e} \pm 59$	$600^{d} \pm 27$	$2650^{d} \pm 110$	$883^{d} \pm 36$	$512^{e} \pm 22$
Site # 3	$1190^{b} \pm 50$	$2116^{a} \pm 95$	$925^{b} \pm 38$	$2910^{a} \pm 135$	$950^{c} \pm 40$	$718^{c} \pm 28$
Site # 4	$958^{d} \pm 42$	$1560^{d} \pm 55$	$500^{e} \pm 22$	$1878^{e} \pm 88$	$703^{e} \pm 28$	$800^{b} \pm 32$
Site # 5	$1370^{a} \pm 58$	$1813^{c} \pm 75$	$1120^{a} \pm 50$	$2732^{c} \pm 120$	$1021^{b} \pm 88$	$608^{d} \pm 30$
Site # 6	$490^{f} \pm 20$	$1020^{f} \pm 45$	$300^{f} \pm 12$	$1425^{f} \pm 58$	$507^{f} \pm 21$	$328^{f} \pm 10$

*Values are mean (n = 3) ± SD; Y = −3.330X + 35.012, correlation coefficient r = 0.996, PCR efficiency: 99.7%.
‡ND: not detected.
†No amplification was observed when 5 μL DNA containing 10^4 genomic equivalents of *Escherichia coli* ATCC 25922 lacking bla_{TEM} gene used as negative control or 5 μL sterile Milli-Q® (Millipore, Billerica, MA, USA) water used as no template control in qPCR assay designed to quantify bla_{TEM}.
§Food article wise one way ANOVA in complete randomized block design significant at 5% level. Different superscripts on means denote significant ($p < 0.05$) difference between the means for one food article according to Duncan's Multiple Range Test.
¶Sampling locations: Site # 1, Aliganj; Site#2, Indira nagar; site #3, Gomtinagar; site # 4, Sadar Bazar; site # 5, Rajajeepurum; site # 6, Alambagh.

environmental reservoirs of bla_{TEM} gene copies harboring bacteria. It has been reported that raw fruits, vegetables, animal meat and other processed food articles were frequently associated with β-lactamase resistant bacteria harboring antimicrobial resistance genes [29,30,37,38].

3.5. Quantitative microbial risk assessment (QMRA) approach to determine risk of infection posed by bacteria exhibiting bla$_{TEM}$ genes

Exposure to surface water contaminated with bacteria exhibiting ARGs pose risk for human health. The infection risk was determined based on best and worst case scenarios. In the best case scenario, where the people were exposed to the least copies of the bla_{TEM} gene (as recorded in the various exposure routes), exposure to surface water of the river Gomti gave the higher risk of infection (4.36×10^{-2}) than the river Ganga. However, in the worst case where the population was exposed to the highest possible concentration of the bla_{TEM} gene copies per 100 mL of the surface water, there was a risk of at least 7 out of 10 people being infected upon exposure (7.21×10^{-1}) to surface water of river Gomti. Therefore, the risk of infection was higher due to exposure to the surface water of river Gomti than for the river Ganga (Table S7 in supplementary materials). The distribution of bla_{TEM} gene in the potable water varies at different sites, however considering the best case scenario (based on gene copies), a risk of 1.89×10^{-3} i.e one person out of a thousand exposed was detected. However, higher concentrations of gene were detected which increases the risk to 7.32×10^{-3} (seven people out of a thousand exposed) in worst case scenario in potable water (Table S8 in supplementary materials). The risk of infection as detected on the basis of bla_{TEM} gene copies in drinking water collected from clinical settings in best case scenario was found to be 8.2×10^{-5} whereas in worst case it was 5.27×10^{-2}. Health risk associated with the consumption of street foods including fruit juices were also assessed (Table S9 in supplementary materials).

The gene copies per 100 mL of different juices collected from study area differs significantly and so as the risk of infections by consumption of these juices. Again assuming best and worst case scenarios, the risk of infections were determined for different fruit juices (Table S9 in supplementary materials).Under the best case scenario Mausammi, Pomegranate and Watermelon juices gave similar risk of infections (10^{-4}), however pineapple, sugarcane and orange juices gave higher risk of infections (10^{-3}). In the case of exposure to high gene copies due to consumption of these fruit juices as reported in this study, there are chances of high risk infections to exposed population. The highest risk under the worst case scenarios was posed by the consumption of sugarcane juice where at least one person out of a hundred will be infected with bacteria with the resistant gene copies (Table S9 in supplementary materials).

In addition to fruit juices the consumption of street foods (contaminated with bacteria exhibiting bla_{TEM} gene) could pose a risk to exposed population. The distribution of bla_{TEM} gene copies in these food items varied significantly, therefore assuming a best case where the least amount of gene copies were recorded, was used for the risk assessment. All food items showed similar risk (10^{-3}) with raw mutton posing the highest risk (8.76×10^{-3}). However under worst case where the highest concentrations were used in the assessment, chowmein and raw mutton pose the highest risk to consumers (10^{-2}), where one out of every hundred people will be infected with bacteria exhibiting bla_{TEM} gene (Table S10 in supplementary materials).

Therefore, environmental reservoirs of TEM-β-lactamase resistance in the river Ganga and its tributaries may initiate epidemic. The quantitative microbial risk assessment (QMRA) was performed for consumption/exposure of surface water collected from both rivers, potable water and street food items including fruit juices to present a picture of risk infection to exposed population. Earlier risk assessment by waterborne pathogens to exposed populations has been reported [39,40].

Hence the quantitative enumeration of bla_{TEM} gene copies in different niches of environment and QMRA approach can provide more reliable and comprehensive information for risk assessment.

4. Conclusions

The present study concludes that a large population comprising local inhabitants and travelers could be exposed to the river water exhibiting bla_{TEM} gene carrying bacteria through aquatic products, drinking water and direct interaction through recreation of religious activities. Besides, environmental reservoirs (surface water, sediment, aquatic flora and vegetables irrigated by water and processed food) exhibiting TEM β-lactamase resistance encoding gene in Indo-Gangetic region pose major risk to the human health. The developed qPCR assay and the QMRA approach could be used in surveillance of TEM β-lactamase resistance in environmental samples and identification of its potential reservoirs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2016.04.036.

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