Environmental Science & Technology

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pubs.acs.org/est

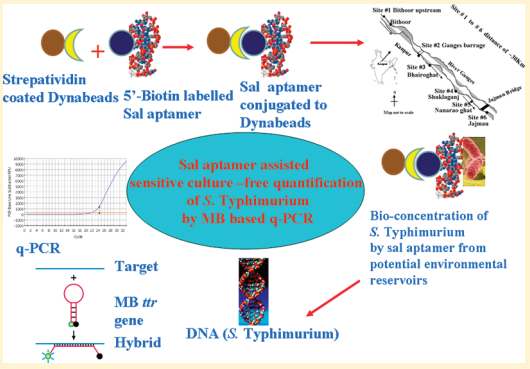
Identification of Environmental Reservoirs of Nontyphoidal Salmonellosis: Aptamer-Assisted Bioconcentration and Subsequent Detection of *Salmonella* Typhimurium by Quantitative Polymerase Chain Reaction

Anurag Jyoti,[†] Poornima Vajpayee,[†] Gulshan Singh, Chandra Bali Patel, Kailash Chand Gupta, and Rishi Shanker*

Environmental Microbiology, CSIR- Indian Institute of Toxicology Research, Post Office Box 80, Mahatma Gandhi Marg, Lucknow-226001, Uttar Pradesh, India

Supporting Information

ABSTRACT: In this study, identification of environmental reservoirs of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (abbreviated as *Salmonella* Typhimurium) in sediments, water, and aquatic flora collected from the Ganges River (Ganges riverine material) was carried out by adopting a two-step strategy. Step 1 comprised a selective serovar-specific capture of *Salmonella* Typhimurium from potential reservoirs. Step 2 involved culture-free detection of selectively captured *Salmonella* Typhimurium by *ttr* gene-specific molecular beacon (MB) based quantitative polymerase chain reaction (q-PCR). The *ttr* gene-specific MB designed in this study could detect 1 colony-forming unit (cfu)/PCR captured by serovar-specific DNA aptamer. Sediments, water, and aquatic flora collected from the Ganges River were highly contaminated with *Salmonella* Typhimurium. The preanalytical step in the form of serovar-specific DNA aptamer-based biocapture of bacterial cells was found to enhance the sensitivity of the fluorescent probe in the presence of nonspecific DNA . Information about the presence of environmental reservoirs of *Salmonella* Typhimurium in the Ganges River region may pave the way for forecasting and management of nontyphoidal salmonellosis in south Asia.



INTRODUCTION

Environmental reservoirs are locations outside the human body within the niche favoring bacterial persistence and replication in the environment and pathogen transmission to susceptible hosts.[1] In Asia and other parts of the developing world, natural surface water is being used by a large population for drinking and other domestic purposes.[2,3] Furthermore, the prevalence of environmental reservoirs in riverine materials and contamination of surface water due to the addition of untreated sewage from nearby residential areas sets them at high risk for water-borne diseases caused by bacterial pathogens.[1] *Salmonella* frequently occurs in sewage-impacted fresh and marine water environments; it causes gastroenteritis and typhoid in humans worldwide, resulting in a high economic burden due to a substantial increase in medical treatment expenditure.[4,5] *Salmonella enterica*, which causes salmonellosis in humans and animals worldwide, is classified into more than 2400 *Salmonella* serotypes.[6] *Salmonella enterica* subsp. *enterica* serovar Typhimurium (abbreviated as *Salmonella* Typhimurium), an important food and freshwater contaminant encountered frequently in most geographical regions, dominates the global epidemiology of *Salmonella*.[7] The infectious dose of *Salmonella* Typhimurium lies between 1 and 10 colony-forming units (cfu).[8] Out of the 1.3 billion incidents of human nontyphoidal salmonellosis reported annually worldwide, approximately 3 million cases end with the patient's death from the disease.[9] However, the mortality rate in developed countries (2%) is less than in resource-constrained countries (18−24%). Agricultural runoff exhibiting poultry waste, bovine manure, and feces of other asymptomatic animals is an important carrier of *Salmonella* Typhimurium to riverine materials. In addition, agricultural produces from crops irrigated by contaminated water have also been associated with a large number of outbreaks.[10] Hence, the presence of *Salmonella* Typhimurium in environmental reservoirs, even in low numbers, may cause a public health risk of nontyphoidal salmonellosis. Conventional culture-based protocols for detection and quantification of *Salmonella* Typhimurium in environmental samples require up to 5 days to obtain a confirmed result and are executable only in samples where the pathogen registers its presence in very high numbers, that is, 10^2-10^3 cfu/g or 10^2-10^3 cfu/mL.[11,12] Quantitative polymerase

Received: June 3, 2011
Accepted: August 29, 2011
Revised: August 24, 2011
Published: August 29, 2011

Table 1. Selectivity of DNA Aptamer for *Salmonella* Typhimurium[a]

Salmonella serovars	detection of *Salmonella* Typhimurium: threshold cycles (C_T)[b]	
	A[c]	B[d]
Salmonella Typhi MTCC 733[c]	29.77 ± 0.988	ND[e]
Salmonella Paratyphi A MTCC 735[c]	30.14 ± 1.245	ND[e]
Salmonella Infantis MTCC 1167[c]	30.77 ± 1.132	ND[e]
Salmonella Typhimurium ATCC 14028[d]	29.89 ± 1.027	29.69 ± 0.875
Salmonella Typhimurium ATCC 13311[d]	29.97 ± 0.978	29.30 ± 0.998
sterile Milli-Q water[f]	ND[e]	ND[e]

[a] 10^6 cells/mL or 10^4 cells/PCR. [b] Each assay was conducted with five independent replicates on the same day, and values are mean C_T ± standard deviation (SD). [c] DNA was prepared directly from spiked water samples without bioconcentration of *Salmonella* Typhimurium cells by Sal aptamer capture of *Salmonella* Typhimurium cells. [d] *Salmonella* Typhimurium cells were bioconcentrated from spiked water samples by serovar-specific DNA aptamer (Sal aptamer) prior to DNA extraction. [e] ND = C_T not detected. [f] Sterile Milli-Q (Millipore, Billerica, MA) served as negative control.

chain reaction (q-PCR) assays targeting the *invA* gene (present in a wide range of *Salmonella* serotypes, including all subspecies, and absent in other bacterial species and genera) offers several advantages compared to classical bacteriology in terms of speed, detection limit, potential for automation, and cost.[7,12−14] However, it has been documented that some *Salmonella* strains exhibit natural deletions within *Salmonella* pathogenicity island 1, where the *inv*, *spa*, and *hil* loci occur.[15] Therefore, q-PCR assays targeting the *invA* gene could lead to false negative results for such strains.[16] Besides, the sensitivity of q-PCR reported for detection of *Salmonella* from water declines 10-fold in the presence of nonspecific DNA.[7] Further, these culture-free assays provide information about total *Salmonella* concentration and fail to detect serovar-specific contamination in environmental samples. Therefore, there is a need for selection of a target that is highly genetically stable in all *Salmonella* strains. Furthermore, the inclusion of preanalytical sample cleanup steps, consisting of selective serovar-specific capture of target pathogen to improve the sensitivity of q-PCR-based assays for culture-free detection of *Salmonella* serovars from sewage-impacted surface water and other complex environmental matrices, is required. Aptamers can be selected for a wide range of targets, including bacterial pathogens, through Systematic Evolution of Ligands by EXponential enrichment (SELEX), which involves iterative cycles of absorption, recovery of bound DNA/RNA, and amplification from a combinatorial DNA/RNA library containing a central randomized sequence and flanking fixed sequences (primers) on both sides for amplification.[17,18] Recently, it has been reported that aptamers can selectively capture and concentrate *Salmonella* Typhimurium from spiked chicken feces.[19] However, aptamers have not been used yet for capture and selective concentration of any *Salmonella* serovar from samples retrieved from a natural environment contaminated by untreated sewage. The *ttr* locus, comprising five genes (*ttrA*, *ttrB*, and *ttrC*, which encode the tetrathionate reductase structural proteins, and *ttrS* and *ttrR*, which encode the sensor and response regulator components of a two-component regulatory system) responsible for tetrathionate respiration, is genetically stable in all *Salmonella* strains.[16] Hence, in this study, identification of environmental reservoirs of *Salmonella* Typhimurium in Ganges riverine samples was carried out by adopting a two-step strategy. Step1 comprised a selective serovar-specific capture of *Salmonella* Typhimurium from potential environmental reservoirs. Step 2 involved culture-free detection of selectively captured *Salmonella* Typhimurium by a *ttr* gene-specific molecular beacon (MB) based q-PCR, designed in a highly conserved region of the *Salmonella*-specific *ttr* locus.

MATERIALS AND METHODS

Bacterial Strains. *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 (abbreviated as *Salmonella* Typhimurium) and *Escherichia coli* DH5α ATCC 35218 were procured from the American Type Culture Collection (ATCC). However, *Escherichia coli* MTCC 723, *Salmonella* Paratyphi A MTCC 735, *Salmonella* Typhi MTCC 733, and *Salmonella* Infantis MTCC 1167 were procured from the Microbial Type Culture Collection at the Institute of Microbial Technology, Chandigarh, India.

Aptamer, Primers, and Molecular Beacon. A DNA aptamer (Sal aptamer: biotin 5′-TATGGCGGCGTCACCCGACGGGGACTTGACATTATGACAG-3′) reported to capture *Salmonella* Typhimurium from spiked chiken feces[19] was biotinylated at the 5′ end to bioconcentrate *Salmonella* Typhimurium from sewage-impacted surface water. Primers (forward primer ttr6, 5′-CTCACCAGGAGATTACAACATGG-3′ , position 4287−4309 bp; reverse primer ttr4, 5′-AGCTCAGACCAAAAGTGACCATC-3′, position 4359−4381 bp; product length 95 bp) specific for the *ttr* gene were adopted from Malorny et al.[16] These primers are located in a highly conserved region of the *Salmonella*-specific *ttr* locus and were designed on the basis of a multiple alignment of the *ttrBCA* sequences.[16] After multiple alignment of the *ttrBCA* sequences, a molecular beacon (5′-CCAGGCGACCGACTTTTAGCCACTGACGAGCCTGG-3′) targeting the *ttr* gene corresponding to adopted primers was designed in the *ttr* locus at the *ttrA* gene (position 4323−4345 bp) for culture-free quantitative enumeration of *Salmonella* Typhimurium to identify environmental reservoirs present in Ganges riverine materials impacted by sewage discharge (Supporting Information). The primers, probe, and DNA aptamer were purchased from MetaBion GmbH, Germany.

Selectivity of DNA Aptamer for *Salmonella* Typhimurium. To test the selectivity of the Sal aptamer for bioconcentration of *Salmonella* Typhimurium, several representative serovars of *Salmonella* were selected (Table 1). Cultures of the reference strains of these serovars grown in Luria−Bertani broth to 0.8 optical density, corresponding to 10^9 cells/mL. Two sets of 10 mL of sterile water (Milli-Q, Millipore, Billerica, MA) were spiked with 10^6 cfu/mL of each reference strain to get 10^4 cfu/PCR in a 25 μL reaction. Cultures were centrifuged at 8000*g* for 4 min (4 °C). Cells were washed three times with 1× phosphate-buffered saline (PBS) and finally, resuspended in 5 mL of 1× PBST (1× PBS contains 1.47 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, and 4.3 mM Na_2HPO_4, pH 7.4; 1× PBST additionally contains 0.5% Tween-20, v/v). Aptamer-conjugated Dynabeads (300 μL) were mixed in resuspended cells and incubated for 30 min at room temperature. Dynabeads were concentrated and supernatant was discarded. Dynabeads were washed 10 times with 1× PBST. Finally, beads were resuspeneded in 250 μL of PBS and stored at 4 °C for further processing. DNA template was

prepared by boiling the Dynabeads suspended in 250 μL of PBS. Supernatant was collected through aspiration by pipet after concentration of Dynabeads in a concentrator. The cellular debris was removed by centrifugation at 16000*g* for 5 min at 4 °C. DNA was precipitated from supernatant by use of sodium acetate (0.3 M, pH 5.2) and ice-cold ethanol.[7] The precipitated DNA was pelleted by centrifugation at 12000*g* for 5 min. DNA pellet was washed three times with 70% ethanol and finally dissolved in 50 μL of TE buffer [10 mM tris(hydroxymethyl) aminomethane (Tris) and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0]. DNA was prepared directly from another set of 10-fold diluted culture without Sal aptamer bioconcentration. The quantity and quality of extracted DNA was measured at 260/280 nm with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA (5 μL) was used as template in a real-time q-PCR assay for detection and quantitative enumeration of *Salmonella* Typhimurium. Each assay was conducted with five independent replicates on the same day, and values are mean $C_T \pm$ standard deviation (SD).

Bioconcentration of *Salmonella* Typhimurium from Potential Environmental Reservoirs for Culture-Free Detection. An Indian perennial river (Ganges River, Kanpur city, latitude 26.28 N; longitude 80.24 E; altitude 126 m; flowing through northern India) was selected to test the applicability of Sal aptamer for bioconcentration of *Salmonella* Typhimurium from potential environmental reservoirs and subsequent detection through culture-free MB based q-PCR assay. The Ganges River receives untreated sewage generated by nearby residential setup of urban population in the boundaries of the Kanpur and Unnao cities.[20] Replicate (n = 5) grab surface water samples (1 L) were collected in sterilized bottles from six sites (Figure S2, Supporting Information) in the Ganges River covering a 30 km stretch (site 1, Bithur upstream; site 2, Ganges barrage; site 3, Bhairoghat (cremation ghat); site 4, Shuklaganj; site 5, Nanaraoghat; site 6, Jajmau). Surface sediments (0−5 cm, ∼250 g) were collected at each sampling location (n = 5) from river bed (∼1 m distance) close to the banks with a stainless steel scoop and placed in plastic bags. Aquatic plants/algae growing any at the selected sites were also collected and placed in polythene bags. The samples were collected on the same day, transported on ice, and analyzed immediately after arrival in the laboratory. Water/sediment and aquatic flora (n = 5 each) were also collected from a pristine site negative for *Salmonella* contamination. Two sets (each comprising five replicates) of 500 mL aliquots of each water sample from each site were concentrated to 5 mL by repeated centrifugation at 18000*g* for 10 min (4 °C). Cells were pelleted from concentrated samples by centrifugation (8000*g* for 4 min at 4 °C) and finally resuspended in 1× PBS (5 mL) after three successive washings by same buffer. Similarly, two sets (each comprising five replicates) of 10 g sediment sample collected from each site were dissolved in 100 mL of phosphate-buffered saline. Bacteria were released by vigorous shaking (10 min at 220 rev·min^{-1}) on a refrigerated rotary shaker (Innova 4230, New Brunswick, NJ). Supernatant from each set of samples was collected separately and concentrated to 5 mL by repeated centrifugation at 18000*g* for 10 min (4 °C). Cells were pelleted from concentrated samples by centrifugation (8000*g* for 4 min at 4 °C) and finally resuspended in 1× PBST (5 mL) after three successive washings by the same buffer. Plant samples (roots/leaf/algal mat) collected from each site including the pristine site were processed for releasing the bacteria adhered to the plants as described by Ram et al.[21] In brief, bacteria adhering to each plant sample (10 g) were released in 100 mL of phosphate-buffered saline through repeated washing by vigorous shaking (2 min at 220 rev·min^{-1}) on a refrigerated rotary shaker (Innova 4230, New Brunswick, NJ) and 45 s of centrifugation at 2000 rpm (653*g*). Two sets of each sample aliquot (100 mL) of phosphate-buffered saline containing bacteria released from plants and 100 mL of each surface water sample collected from each site in the Ganges River were concentrated to 5 mL by repeated centrifugation at 18000*g* for 10 min (4 °C). Cells were pelleted from concentrated samples by centrifugation (8000*g* for 4 min at 4 °C) and finally resuspended in 1× PBST (5 mL) after three successive washings in 1× PBS.

One set of environmental samples (plant/water/sediment) was subjected to bioconcentration of *Salmonella* Typhimurium by use of the Sal aptamer from water samples containing various pathogenic and nonpathogenic bacteria. Briefly, 5 mL concentrated samples were exposed to 0.4 nM aptamer conjugated to Dynabeads (suspended in 300 μL of 1× PBST). Samples were incubated for 30 min at room temperature. Dynabeads were concentrated on a magnetic particle concentrator (Dyna Mag^{TM} 2, Invitrogen), and DNA was prepared as described in Supporting Information.

The other set of samples (plant/water/sediment), comprising cells suspended in 5 mL of 1× PBS, was processed for isolation of multigenomic DNA through boiling prep as described by Jyoti et al.[7] Briefly, DNA template was prepared by boiling 500 μL of concentrated water and removing the debris by centrifugation at 16000*g* for 5 min at 4 °C. DNA was precipitated from supernatant by use of sodium acetate (0.3 M, pH 5.2) and ice-cold ethanol.[7] The precipitated DNA was pelleted by centrifugation at 12000*g* for 5 min. The DNA pellet was washed three times with 70% ethanol and finally dissolved in 250 μL of TE buffer. DNA (5 μL) was used as template in 25 μL q-PCR assays (Supporting Information). Quantitative enumeration of *Salmonella* Typhimurium at each sampling location in potential environmental reservoirs was carried out by use of a standard curve prepared from 10-fold diluted genomic DNA of *Salmonella* Typhimurium ATCC 14028 (from 1×10^6 down to 1×10^0 genomic equivalent (GE)/PCR).

Statistical Analysis. 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 real-time detection system software, version 3.0A. Amplification efficiency (E) was estimated by using the slope of the standard curve and the formula $E = [10^{(-1/\text{slope})}]-1$. A reaction with theoretical 100% efficiency will generate a slope of −3.322. The comparison of pollution level of six sites in the Ganges River, in terms of *Salmonella* Typhimurium load, was performed by one-way analysis of variance (ANOVA).[22] Duncan's multiple range test was used to compare the means.[23]

RESULTS

Sensitivity and Specificity of the *ttr* Gene-Targeting Molecular Beacon. q-PCR in the molecular beacon format described here is highly sensitive and specific to *Salmonella* species. The five reference strains and 15 isolates of *Salmonella* retrieved from potable water collected from a major city of northern India were positive for the *ttr* gene (Table S1, Supporting Information). However, no amplification of the *ttr* gene was observed in *E. coli* and *Enterococcus* reference and environmental isolates, lacking the target gene with the assay developed in this study (Table S1, Supporting Information). The detection probability

Table 2. Contamination of Ganges River by *Salmonella* spp. and *Salmonella* Typhimurium[a]

	quantitative enumeration of *Salmonella* spp. and *Salmonella* Typhimurium in Ganges River[b]			
	water		sediment	
sites[c]	*Salmonella* spp. per 100 mL[d]	*Salmonella* Typhimurium per 100 mL[e]	*Salmonella* spp. per 100 g[d]	*Salmonella* Typhimurium per 100 g[e]
site 1	$(2.62 \times 10^3) \pm (8.8 \times 10^1)$ f	$(2.37 \times 10^2) \pm (1.1 \times 10^1)$ f	$(4.37 \times 10^4) \pm (2.05 \times 10^2)$ f	$(3.37 \times 10^4(\pm (1.50 \times 10^2)$ f
site 2	$(3.46 \times 10^4) \pm (1.09 \times 10^2)$ e	$(1.96 \times 10^3) \pm (8.8 \times 10^1)$ e	$(5.62 \times 10^5) \pm (1.40 \times 10^4)$ e	$(1.96 \times 10^4) \pm (1.02 \times 10^2)$ e
site 3	$(3.99 \times 10^9) \pm (1.60 \times 10^7)$ a	$(2.08 \times 10^8) \pm (9.7 \times 10^6)$ a	$(8.51 \times 10^9) \pm (1.57 \times 10^6)$ a	$(3.08 \times 10^9) \pm (9.7 \times 10^6)$ a
site 4	$(4.68 \times 10^5) \pm (1.02 \times 10^4)$ d	$(2.26 \times 10^3) \pm (8.0 \times 10^1)$ d	$(7.68 \times 10^6) \pm (2.69 \times 10^5)$ d	$(3.26 \times 10^4) \pm (1.00 \times 10^2)$ d
site 5	$(4.51 \times 10^8) \pm (1.57 \times 10^7)$ b	$(6.64 \times 10^6) \pm (6.52 \times 10^4)$ b	$(4.67 \times 10^9) \pm (6.64 \times 10^6)$ b	$(7.64 \times 10^7) \pm (6.98 \times 10^4)$ b
site 6	$(2.67 \times 10^8) \pm (6.64 \times 10^6)$ c	$(3.35 \times 10^6) \pm (8.67 \times 10^4)$ c	$(3.99 \times 10^9) \pm (1.60 \times 10^7)$ c	$(5.35 \times 10^6) \pm (8.67 \times 10^4)$ c

[a] *Salmonella* Typhimurium is a nontyphoidal salmonellosis-causing serovar. [b] Values are mean ($n = 5$) ± SD. Sterile Milli-Q water (Millipore, Billerica, MA) was used as negative control. Column-wise one-way ANOVA, performed separately, was significant at 5% level for each column. Lowercase roman letters show significant difference between means in a column according to Duncan's multiple range test. [c] Site 1, Bithoor upstream; site 2, Ganges barrage; site 3, Bhairoghat; site 4, Shuklaganj; site 5, Nanaraoghat; site 6, Jajmau. [d] DNA was prepared directly from water and sediment samples without Sal aptamer bioconcentration for *Salmonella* Typhimurium cells. [e] *Salmonella* Typhimurium cells were bioconcentrated from water and sediment samples by serovar-specific DNA aptamer (Sal aptamer) prior to DNA extraction.

of the developed assay was 100% for 1 cfu/PCR (Table S2, Supporting Information). However, the presence of background DNA from *E. coli* DH5α and enterotoxigenic *E. coli* (*Escherichia coli* MTCC 723;10^6 cfu/PCR each) dropped the sensitivity of the assay 1000-fold (Table S4, Supporting Information).

Conjugation of Aptamer to Dynabeads and Binding of *Salmonella* Typhimurium to Aptamer-Conjugated Dynabeads. The observations indicate that approximately 96.75% of the Sal aptamer was conjugated to the streptavidin-coated Dynabeads (Table S3, Supporting Information).

Selectivity of DNA Aptamer for *Salmonella* Typhimurium. The DNA aptamer is highly selective for the *Salmonella* Typhimurium (Table 1) . No amplification was recorded when *Salmonella* cells of other serovars were bioconcentrated by the Sal aptamer. However, the MB designed in this study could detect all the *Salmonella* reference strains tested in this study. The selected DNA aptamer could concentrate only *Salmonella* Typhimurium and was found to be highly specific to the serovar.

Bioconcentration of *Salmonella* Typhimurium from Water Spiked with Reference Strain in the Presence of Background Bacterial Flora. Aptamer-conjugated Dynabeads could bioconcentrate *Salmonella* Typhimurium from water samples spiked with 10-fold diluted culture of *Salmonella* Typhimurium ATCC 14028 as Dynabeads plated on HiCrome improved salmonella agar showed characteristic pink colonies (Figure S1, Supporting Information). The lowest cell concentration captured by aptamer-conjugated Dynabeads and detected by *ttr* gene-targeted MB-based q-PCR was 1 cfu/PCR from water samples spiked with *Salmonella* Typhimurium (Table S4, Supporting Information). Further, Sal aptamer was able to bioconcentrate *Salmonella* Typhimurium cells from the spiked water in the presence of a high background of pathogenic and nonpathogenic bacteria without any drop in detection limit due to presence of nonspecific DNA (Table S4, Supporting Information). Our observations indicate that the detection limit of the *ttr* gene-targeted *Salmonella* specific q-PCR drops 1000-fold in the presence of mixed background of *E. coli* DH5α (10^8 cfu/mL or 10^6 cfu/PCR) and enterotoxigenic *E. coli* (ETEC; 10^8 cfu/mL or 10^6 cfu/PCR).

Bioconcentration of *Salmonella* Typhimurium from Potential Environmental Reservoirs and Subsequent Culture-Free Detection. All the water and sediment samples collected from the Ganges River were found to be contaminated by *Salmonella* (Table 2). However, the level of the *Salmonella* contamination varied significantly (Duncan's multiple range test, $p < 0.05$) among the sampling locations (Table 2). Water and sediment samples collected from site 3 exhibit a maximum level (water, 3.99×10^9 cfu/100 mL; sediment, 8.51×10^9 cfu/100 g) of *Salmonella*. However, a minimum level (water, 2.62×10^3 cfu/100 mL; sediment, 4.37×10^4 cfu/100 g) of *Salmonella* contamination in both water and sediment of the Ganges River observed at site 1 (Table 2).

Furthermore, it has been observed that *Salmonella* Typhimurium contamination in water samples collected from selected sites ranged between 2.37×10^2 and 2.08×10^8 cfu/100 mL. Sediments from the selected sampling locations also exhibit *Salmonella* Typhimurium in the range of 3.37×10^4 to 3.08×10^9 cfu/100 g (Table 2).

The level of the *Salmonella* Typhimurium was highest at site 3. The inclusion of a preanalytical step in the form of cell capture by the serovar- (*Salmonella* Typhimurium) specific aptamer allowed enumeration of the *Salmonella* Typhimurium contamination. Sediments were more contaminated than water (Table 2). Aquatic flora collected from the Ganges River were also contaminated by *Salmonella* Typhimurium (Table 3). *Potamogeton crispus* exhibited maximum *Salmonella* and *Salmonella* Typhimurium contamination, followed by *Potamogeton pectinatus* and *Spirogyra* spp. (Table 3).

DISCUSSION

The detection of low levels of pathogenic bacteria in complex sample matrices such as environmental samples containing numerous polymerase chain reaction inhibitors is challenging due to the drop in sensitivity of rapid molecular fluorescent probe-based techniques.[7,16,18] The sensitivity of the *invA* gene-targeted MB-based q-PCR reported by Jyoti et al.[7] dropped 10-fold in the presence if 10^6 cfu/PCR *E. coli* DH5α ATCC 35218. In this study, we observed that addition of enterotoxigenic *E. coli* (10^6 cfu/PCR) along with *E. coli* DH5α ATCC 35218 (10^6 cfu/PCR) decreased the sensitivity of the *ttr* gene-specific MB-based q-PCR assay 1000-fold. Therefore, it could be extrapolated that the assay may perform poorly in environmental samples where multigenomic DNA is present. Also, the assay reported by Jyoti et al. [7] and other fluorescent probe-based assays could quantify only levels of the genus *Salmonella*.[7,12−14] Further, these assays fail to provide

Table 3. Total *Salmonella* and *Salmonella* Typhimurium Contamination in Potential Environmental Reservoirs of the Ganges River at Kanpur

	quantitative enumeration of *Salmonella* (cfu/100 g)[a]	
aquatic flora	*Salmonella* spp.[b]	*Salmonella* Typhimurium[c]
	Potamogeton pectinatus[d]	
sample 1[e]	55 360 ± 190 c	1384 ± 50 c
sample 2[f]	68 990 ± 207 b	3725 ± 130 b
sample 3[g]	487 999 ± 19201 a	8958 ± 376 a
	Potamogeton crispus[d]	
sample1[e]	85 365 ± 341 c	3172 ± 142 c
sample 2[g]	785 289 ± 23 558 a	10 856 ± 379 a
sample 3[h]	98 259 ± 365 b	5436 ± 190 b
	Spirogyra spp.[d]	
sample 1[e]	67 251 ± 326 d	4529 ± 262 d
sample 2[f]	82 821 ± 4126 c	7523 ± 300 c
sample 3[i]	282 652 ± 11 306 a	46 689 ± 2120 a
sample 4[j]	125 689 ± 6081 b	25 826 ± 1033 b

[a] Values are mean (n = 5) ± SD. Sterile Milli-Q water (Millipore, Billerica, MA) was used as negative control in all experiments. Column-wise one-way ANOVA, performed separately for each aquatic macrophyte and for *Spirogyra* spp., was significant at 5% level. Lowercase Roman letters in each column for each aquatic macrophyte and for *Spirogyra* spp. show significant difference between means according to Duncan's multiple range test. [b] DNA was prepared directly from water and sediment samples without Sal aptamer bioconcentration for *Salmonella* Typhimurium cells. [c] *Salmonella* Typhimurium cells were bioconcentrated from water and sediment samples by serovar-specific DNA aptamer (Sal aptamer) prior to DNA extraction. [d] Collected from a pristine area that exhibited no *Salmonella* contamination. [e] Site 1, Bithur upstream. [f] Site 2, Ganges barrage. [g] Site 3, Bhairoghat (cremation ghat). [h] Site 4, Shuklaganj. [i] Site 5, Nanaraoghat. [j] Site 6, Jajmau.

serovar-specific information on *Salmonella* contamination in the environmental samples. Therefore, sensitive and robust preanalytical sample processing tools that facilitate separation and concentration of the target organism for subsequent detection and quantification by methods such as florescence probe-based q-PCR are needed. In this study, the application of a DNA aptamer to bioconcentrate the selected serovar of *Salmonella* Typhimurium from environmental samples to identify potential environmental reservoirs of *Salmonella* Typhimurium prior to culture-free detection of *Salmonella* Typhimurium through MB-based q-PCR was explored for the first time. The assay reported in this study was able to bioconcentrate *Salmonella* Typhimurium from potential environmental reservoirs exhibiting low concentrations of *Salmonella* Typhimurium, which was subsequently detected by the *Salmonella*-specific highly conserved region of the *ttr* gene-targeted MB-based q-PCR (lowest detection limit was 1 cfu/PCR). It has been observed that preanalytical bioconcentration of *Salmonella* Typhimurium increased the sensitivity of the MB-based q-PCR assay by selective capture of the target cells from samples spiked with reference strain (lowest detection limit was 1 cfu/PCR in the presence of high background microflora). We have tested our assay by analyzing environmental samples collected from a sewage-impacted perennial river, the Ganges River flowing through northern India, that provides water for drinking and other domestic requirements to a large population in South Asia and exhibits high endemicity for *Salmonella* contamination.[20,24]

In this study, we have observed high levels of *Salmonella* and *Salmonella* Typhimurium contamination in potential environmental reservoirs (the green alga *Spirogyra*, the aquatic macrophytes *P. crispus* and *P. pectinatus*, and river bed sediments) in riverine materials from the Ganges River. Earlier, it was observed that *Cladophora* in Lake Michigan serves as an environmental reservoir for *Salmonella* and potentially other pathogens, such as shiga toxin-producing *E. coli*, *Shigella*, and *Campylobacter*.[25] However, that study accounts for *Salmonella* contamination and lacks serovar-specific information. Recently, the presence of *Enterococcus* and enterotoxigenic *E. coli* has also been reported in submerged macrophytes and sediments collected from stream and river banks.[26,27]

Furthermore, it has been reported that elevated concentrations of fecal indicator bacteria in aquatic sediments and vegetation have prompted concern that environmental reservoirs of fecal indicator bacteria disrupt the correlation between indicator organisms, pathogens, and human health risks.[27] Environmental reservoirs of a pathogenic bacterium could disseminate pathogen across large geographical boundaries and act as vectors of infection.[1,26] Therefore, environmental reservoirs of *Salmonella* Typhimurium in riverine materials from the Ganges River can initiate epidemic and may be critical to the disease's endemicity. It has been suggested earlier that, in a country where cholera is endemic, ingestion of water from ponds or rivers during plankton blooms provide the requisite infectious dose for clinical cholera.[28] Therefore, identification of environmental reservoirs of *Salmonella* Typhimurium is important for defining the potential role of these reservoirs in the transmission pathways of nontyphoidal salmonellosis leading to human illness and is critical to further reduce disease burden, including management of the disease in the endemic areas. Further, quick detection and quantification of *Salmonella* Typhimurium in potential environmental reservoirs may limit the morbidity and mortality of nontyphoidal salmonellosis in high-endemicity zones like India in south Asia.

Salmonella Typhimurium is the most frequently reported serotype of clinical importance and predominates over other serotypes in environmental studies due to its high survival rate outside the host.[5,29] Hence, high concentration of *Salmonella* Typhimurium in river water and potential environmental reservoirs widespread in the riverine system of the Ganges River is possibly attributed to addition of untreated sewage, hospital wastes of medical college and several other clinical setups, poultry wastes, rain storm waters containing fecal pollutants from nearby unauthorized human settlements, agricultural runoff, and the ability of *Salmonella* Typhimurium to survive in the environment outside the host.[20] Increased detection limits in the presence of high background flora by bioconcentration of *Salmonella* Typhimurium enhanced the applicability of the developed MB-based q-PCR assay in complex matrices containing PCR inhibitors. The present study for the first time provides quantitative information about *Salmonella* Typhimurium in the high-endemicity zone of *Salmonella* in south Asia by use of sensitive culture-free protocols involving preanalytical bioconcentration of *Salmonella* Typhimurium. India, Indonesia, Bangladesh, and Pakistan have been identified as high-risk sites for infections caused by *Salmonella* species.[24,30,31]

In conclusion, it could be inferred from the present study that the inclusion of a preanalytical step, in the form of pathogen-specific DNA aptamer-based bioconcentration of the bacterial cell, enhanced the sensitivity of fluorescent probe-based q-PCR

assays. Furthermore, information about the contamination of surface water by *Salmonella* Typhimurium in Ganges riverine materials may pave the way for forecasting and management of nontyphoidal salmonellosis in south Asia.

■ ASSOCIATED CONTENT

Supporting Information. Additional text, four tables, and two figures showing design of *ttr* gene-specific MB, standard curve generation, conjugation of *Salmonella* Typhimurium-specific DNA aptamer to Dynabeads, binding of *Salmonella* Typhimurium cells to Sal aptamer-conjugated Dynabeads, enumeration of *Salmonella* Typhimurium in water samples spiked with reference strain in the presence or absence of high background of nonspecific DNA, and a map depicting sampling sites. This material is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 91+ 0522 −2613786/2614118/2627586, ext 237; fax: 91+ 0522-2611547; e-mail: rishishanker1@rediffmail.com or rishishanker@gmail.com.

Author Contributions

†A.J. and P.V. contributed equally to this work.

■ ACKNOWLEDGMENT

This work was supported by CSIR Network Project NWP-17. Financial assistance to A.J. (SRF), G.S. (SRF), C.B.P. (SRF), and P.V. (scientist: WOS-A) from CSIR and DST, Government of India, New Delhi, respectively, is acknowledged. The paper bears IITR number 2949.

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