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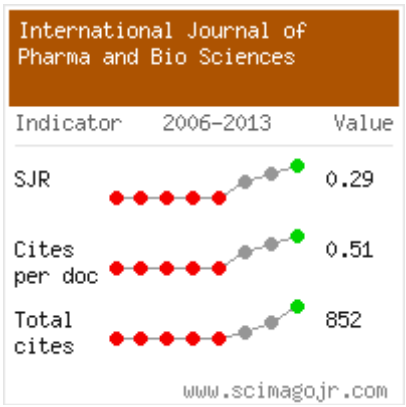

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PREVALENCE OF ANTIMICROBIAL RESISTANCE AND VIRULENCE DETERMINANT OF *SALMONELLAE* ISOLATED FROM POTABLE WATER

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ABSTRACT

The safety of drinking water in an urban city has been challenging since decades. *Salmonellae* are the enteric pathogens which survive even in treated drinking water. The pathogen harbors *invA* signature gene responsible for the virulence. Upon infection with *Salmonellae*, frequent antimicrobial therapy has lead the emergence of drug-resistant strains. The present paper aims to isolate and characterize drug resistant virulent isolates of *Salmonellae* from drinking water. We have collected drinking water from Gwalior, an important city of Northern India. Out of the five sites, three sites were contaminated with *Salmonellae* harboring *invA* gene. All the isolates from sites were resistant against at least 2 antimicrobials. Isolates from site 1, site 2 and site 5 were intermediates (reduced susceptibility) to different antimicrobials. These indicate possibilities of emergence of resistant strains in future. Our observation indicates the presence of drug-resistant isolates in drinking water of Gwalior. This has immense importance for microbial risk assessment.

KEY WORDS: **Potable water, *Salmonellae*, virulence determinants, Antimicrobial resistance**

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INTRODUCTION

The deterioration in the microbiological quality of water adversely impacts human health worldwide. Rapid population growth has placed new challenges on management of the frequency of water-borne disease outbreaks. The majority of water borne infectious diseases that occurs includes diarrhea, typhoid, cholera and others[1]. The over dependence on water has leads to purity of drinking water in developing countries like India[2]. This has also contaminated the natural resources with deadly pathogens like *S.* Typhi. Therefore, identification of critical contamination points for the distribution of drinking water is necessary[3]. Conventional methods for detection rely on the cultivation of specific bacteria in appropriate culture media and on their biochemical characterization. These methods are laborious, non-specific and time consuming [3, 4]. The molecular approach, Polymerase Chain Reaction (PCR) has been validated by the International Organization for Standardization (ISO) and is now used for testing of water and food borne pathogens[5, 6, 7]. The *invA* gene, encodes the invasion associated specialized protein and lies at centisome 63 region of the *Salmonella* chromosome[8]. The *invA* virulent gene is present in almost all the serotype of *Salmonella*. *Salmonella* isolates harbouring *invA* gene have been identified in potable water using molecular methods[9, 10]. Recently, prevalence of *Salmonellae* in surface and drinking water has been demonstrated by a conventional PCR assay targeting *invA* gene[2]. Polymerase Chain Reaction has numerous advantages over conventional methods as they are time consuming, labour intensive, costly and also not specific[5]. A number of studies have been reported for quantification of *Salmonella* in food, vegetables and clinical samples[10, 11, 12]. The occurrence and prevalence of *Salmonella* in potable water is still a major issue. The prevalence of Typhoidal fever is alarming for developing and developed countries[13]. India has been categories under high risk zone for typhoidal caused by *S.* Typhi [14]. A number of outbreaks have been reported due to consumption of *Salmonella* infected food materials [15]. Typhoid fever caused by S. typhi is a major disease in India [16]. The indiscriminate use of antibiotics in clinical infections, easy availability of antibiotics without prescription contributes to the emergence and dissemination of drug resistance in bacteria[17]. In India and other developing countries, pathogen diagnostics based on antimicrobial resistance and virulence gene profiles of *S.* Typhi of potable water resources is not well established. Therefore, antibiotic resistance and plasmid profile of *S.* Typhi isolates recovered from potable waters will be helpful in future therapeutic advancements in humans. With the frequent use of antibiotics to kill *Salmonellae* in previous decades, the pathogen has evolved resistance mechanism to combat against them. As a result, the drug-resistant (DR) *Salmonellae* strains have been prevalent in environment and spread worldwide, resulting in high rates of morbidity and mortality[18]. The extensive use of antibiotics have generated and disseminated drug-resistant *S.* Typhi in the environment and potable water drinking system. The emergence of MDR *S.* Typhi strains to existing antibiotics such as ampicillin, chloramphenicol and co-trimoxazole has complicated the treatment of typhoid fever. In the present work, we report the presence of antimicrobial-resistant *Salmonella* virulent determinants in drinking water samples collected from different sites in Gwalior as a model city.

MATERIALS AND METHODS

Primers

Gene sequences of *invA* were retrieved from NCBI Genbank. The conserved regions, obtained from ClustalW were used to compute primers using dedicated web based software Primer3 (v. 0.4.0). The primer designing parameters such as melting temperature, GC content, amplicon length, etc were taken into consideration. BLAST was used to check the specificity of primers. Further, specificity of the primer set was validated against reference strain *Salmonella typhi* MTCC 733.

Bacterial strains

Reference strains were used to check the specificity of primers as listed in Table 1. The reference strains (*Salmonella typhi* MTCC 733, *Escherichia coli* MTCC 723) were procured from Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology (IMTECH), Chandigarh, India. *Salmonella typhi* MTCC 733 was used as positive control for PCR.

Determination of specificity of the test

The inclusivity and exclusivity of the primer pair was checked using reference strains and environmental isolates of *Salmonella* and other genera. Fifteen bacterial strains have been tested for specificity (Table 1) including one *invA* gene positive reference strain of *Salmonella* (*Salmonella typhi* MTCC 733) 2 reference strains of *Escherichia coli* (*Escherichia coli* MTCC 723 and *Escherichia coli* MTCC 1652) and 12 environmental isolates of *E. coli.* DNA from all the strains was isolated using boiling prep method followed by precipitation and purification using Sodium Acetate (3 M, pH 5.2). The specificity of primers was analyzed using PCR. The PCR reaction mixture in a final volume of 25 µl comprised of dNTPs (0.2 mM), Taq DNA polymerase (1 unit), 10x reaction buffer (2.5 µl), $MgCl_2$ (1.5 mM), primers (0.4 µM, each) for *invA* gene and DNA template (5 µl). The PCR program was done at: initial denaturation at 95^0C for 3 min and then 35 cycles at 95^0C for 20 s, 54.0^0C for 30s, and 72^0C for 30s.

Sample collection

Gwalior is a major and important city of state Madhya Pradesh. Every year in the dry season, the city suffers drinking water supply crisis. Rapid industrialization and population growth has exploited the existing drinking water resources. This has resulted in pollution and deterioration in the microbiological quality of water. The quality of drinking water in the Gwalior is deteriorating day by day. A number of factors are responsible including insufficient treatment plants, direct discharge of untreated sewage into rivers and the faulty and leaky pipe line distribution water system. The antibiotic resistant profiling for appraisal of microbial risk exerted to humans *Salmonella* spp. potable water samples collected from civic drinking water supply. Potable water samples (2 litres) were collected in sterilized bottles from five sites in the Gwalior city. The water samples were collected on the same day, transported on ice and analyzed immediately after arrival in the laboratory.

Isolation and identification of Salmonellae

The samples of each site were filtered through a membrane filter (cellulose nitrate filter of 0.45 µm pore size; Millipore, USA). Each membrane filter was aseptically removed and placed on culture media containing HiCrome Improved Salmonella Agar (Himedia) and incubated overnight at 37^0C. The isolates confirmed as *Salmonella* spp. were maintained at –70°C supplemented with 15% (vol/vol) glycerol.

Detection of virulence determinants specific to Salmonellae

DNA template was prepared from samples by boiling prep method followed by precipitation using sodium acetate (0.3 M, pH 5.2) and followed by ethanol [19]. The precipitated DNA was washed slightly by 70% ethanol and was re-suspended in 100 µl TE (pH 8.0). We selected 3 random *Salmonella* isolates from each site for the presence of signature virulent gene (*invA*) specific to *Salmonella* spp. using primers (Table 1) and cyclic conditions. The PCR reaction mixture in a final volume of 25 µl comprised of dNTPs (0.2 mM), Taq DNA polymerase (1 unit), 10x reaction buffer (2.5 µl), $MgCl_2$ (1.5 mM), primers (0.4 µM, each) for *invA* gene and DNA template (5 µl). The PCR program was as follows: initial denaturation at 95^0C for 3 min and then 35 cycles at 95^0C for 20 s, 54.0^0C for 30s, and 72^0C for 30s followed at 72^0C for final extension for 7 min. All the assays were done in triplicate. Amplicon were analyzed on 1.7% agarose gel containing ethidium bromide (0.5 µg/mL) and were visualized and recorded. We used *Salmonella typhi* MTCC 733 as the positive control for *invA* gene.

Antimicrobial susceptibility test

Three random isolates were selected for screening against antimicrobials from three identified sites. Total nine isolates were used against twelve antibiotics from six different classes as per CLSI guidelines. These are aminoglycosides (amikacin, 10 µg/disc; gentamicin, 10 µg/disc; neomycin, 30 µg/disc; streptomycin, 10 µg/disc); β-lactams (ampicillin, 10 µg/disc); cephalosporins (ceftazidime, 30 µg/disc; cephalothin, 30 µg/disc); folate inhibitors (co-trimoxazole, 25 µg/disc); fluoroquinolones (ciprofloxacin, 5 µg/disc; norfloxacin, 10 µg/disc); phenicols (chloramphenicol, 10 µg/disc) and quinolones (nalidixic acid, 30 µg/disc). Each test was performed in triplicate. Data for antimicrobial resistance of each bacterial isolate have been reported as resistant (R), isolates with reduced susceptibility (RS or I for intermediates) or sensitive (S), based on Clinical and Laboratory Standards Institute break points [20].

RESULTS AND DISCUSSION

Computation of primers and probe for detection of Salmonellae

PCR primers were computed using Primer3 (v. 0.4.0) software. The designed primers were in the range of 18-20 bases (Table 1). Bacterial pathogenic islands harbor a large number of virulence factors, which are essential for the survival and pathogenicity of bacteria. These essential genes are more evolutionary conserved than non-essential genes in bacteria.

Table 1
Primer sequences of invA gene for the detection of Salmonellae

Gene	Primer Sequence	GC (%)	Tm (^{0}C)	Amplicon Length
invA	Forward: 5'-CGCACCGTCAAAGGAACC-3'	61.1	56.8	147 bp
	Reverse: 5'-GCCCGATTTTCTCTGGATGG-3'	55	56	

Occurrence of virulence determinants in Salmonellae isolates

Samples from three sites were positive for the *Salmonellae*. The 147 bp amplicons were observed in these samples on gel electrophoresis after PCR (Figure 1). Results revealed that 60 % of *Salmonellae* strains isolated from the potable water exhibited virulent gene. Remaining isolates were negative for the presence of *invA* gene (Table 2). Our interpretation on virulence markers indicated that the potable water in Gwalior is contaminated by *Salmonellae* isolates exhibiting *invA* gene. The present study indicates that the potable water of Gwalior is contaminated with *Salmonellae* harboring *invA* gene. Contaminated water and poor hygiene are the reasons for diarrheal diseases throughout the world. Fifteen countries contribute three quarters of childhood deaths due to diarrhea in children under five years of age worldwide out of which India ranks first [21]. In India, acute diarrheal diseases lead to 13% deaths in under five age group, during the year 2009, about 11.2 million cases with 1,762 deaths were reported [22]. Presence of typhoidal strains of *Salmonellae* in drinking water supplies is alarming for human health both in rural and urban settings.

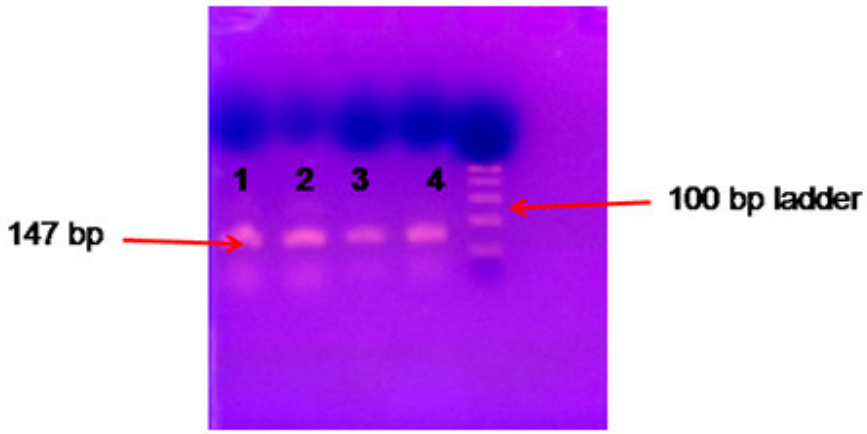

Figure 1
Agarose gel electrophoretic image of amplicons after PCR
1:Salmonella typhi MTCC 733 (Positive Control); 2,3,4: Environmental samples

In present study, we found potable water samples contaminated with strains of *Salmonellae*. The presence of *Salmonellae* in potable water suggests the possibility of contamination of water supplies[19]. Water channels flowing in the old city and other areas are often leaky and unmanaged. As a result they are often in contact with the sewage pipelines. Most of the strains were in culturable state, which shows the presence of high organic matter in the potable water. Still some of the strains may have undergone in VBNC state which could not be cultured. The VBNC state of bacteria has potential to be virulent inside host [23, 24]. A number of reports have been documented for the contamination of potable water pipelines by sewage pipelines [19, 25, 26]. *Salmonellae* isolates from surface water samples from India have been reported to exhibit *invA* and *ttr* genes [16]. The presence of the *invA* gene makes these isolates more virulent. *InvA* is a member of set of several inner membrane proteins that form type three secretion systems which is responsible for invasion of cells. All *Salmonellae* isolates positive for the presence of *invA* gene, have the capacity to invade intestinal cells.

Antimicrobials Susceptibility

Most of the *Salmonellae* isolates in the present study were resistant to at least one antimicrobial (Table 2). Three randomly picked isolates were found to be resistant to more than four antimicrobials. The resistance observed in both Cephlothin and Nalidixic acid is alarming for human health. In similar manner Norfloxacin and Ciprofloxacin resistant isolates were observed. Isolates from site 5 were resistant to atleast three antimicrobials.

Table 2
Antimicrobial resistance and virulence determinants of potential Salmonellae in drinking water

Location	Isolate ID	Antimicrobial resistance	Virulence gene (*invA*)
Site 1	1A	Ne, Ge, Str	+
	1B	Chl	+
	1C	Ne, Ge, Str	+
Site 2	2A	Co, NA, Am, No, Ci, Ne, Ge, Str	+
	2B	Co, NA, Am, Ce, Ci, Cef, Ni, Str, Chl	+
	2C	Nor, Ce, Amp, Cef, Ne, Str	+
Site 3		-	-
Site 4		-	-
Site 5	5A	Co, Ce, Amp, Cef, Ne, Ge, Str, Chl	+
	5B	Ce, Amp, Ne, Str	+
	5C	Am, Amp, Ne	+

Ne: Neomycin, Ge: Gentamicin, Str: Streptomycin, Chl: Chloramphenicol, Co: Co-Trimoxazole, NA: Nalidixic acid, Am: Amikacin, No: Norfloxacin, Ci: Ciprofloxacin, Amp: Ampicillin, Ce: Cefalothin, Cef: Ceftazidime.

Almost all the isolates from three sites were resistant to Streptomycin. Isolates from site 1, site 2 and site 5 were intermediates (reduced susceptibility) to eight, ten and eight different antimicrobials (Table 3). These indicate possibilities of emergence of resistant strains in future.

Table 3
Resistance patterns of Salmonellae isolates from different sites.

		Site 1			Site 2			Site 5		
S. No.	**Antibiotics**	**1A**	**1B**	**1C**	**2A**	**2B**	**2C**	**5A**	**5B**	**5C**
1.	Co-Trimoxazole	S	S	I	R	R	I	R	I	I
2.	Nalidixic acid	S	S	I	R	R	I	I	I	I
3.	Amikacin	I	S	I	R	R	I	I	I	R
4.	Norfloxacin	S	I	I	R	I	R	I	I	I
5.	Cefalothin	I	I	I	I	R	R	R	R	I
6.	Ciprofloxacin	S	S	S	R	R	I	I	I	I
7.	Ampicillin	I	S	I	I	S	R	R	R	R
8.	Ceftazidime	I	S	I	I	R	R	R	I	I
9.	Neomycin	R	I	R	R	R	R	R	R	R
10.	Gentamicin	R	I	R	R	I	I	R	I	I
11.	Streptomycin	R	I	R	R	R	R	R	R	I
12.	Chloramphenicol	S	R	S	S	R	I	R	I	I

R: Resistant, I: Intermediate (Reduced Susceptibility), S: Susceptible

In the present study, a number of isolates exhibited resistance to cephalothin, nalidixic acid and norfloxacin. In general, plasmid harbor antimicrobial resistance gene, which further disseminate among other bacteria through horizontal gene transfer [27, 28]. The resistant strains are highly important as they possess resistance against the antimicrobials which are used in clinics[29, 30]. This has consequence of spread and the emergence of antibiotic resistance among other pathogenic isolates. Some of the isolates were intermediates for the resistance against antibiotics. These isolates can emergence as the resistant ones in near future [31, 32, 33].

CONCLUSION

The present study demonstrates the prevalence of antimicrobials resistance and intermediates isolates of *Salmonellae* in potable water of Gwalior. The presence of pathogenic strains of *Salmonellae* is alarming which may be the major roadblocks for the management of water-borne outbreaks. The study could be used for surveillance of potable water resources and

development of risk assessment strategies for protection of public health.

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