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Isolation of *Salmonella* Typhimurium and Detection of Virulence Contributing Genes from Water of River Ganges

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Abstract: River Ganges is one of the most important rivers of India. This important and sacred river is becoming contaminated day by day. In the present study, 500 water samples were collected from river Ganges and screened for presence of *Salmonella* by PCR. Out of 500 samples, 44 were found to be positive for *Salmonella* and *Salmonella enterica* serovar Typhimurium was the most common serovar isolated from 24 samples. These isolates were screened for presence of *stn, inv* A and *pef* A genes which are mainly responsible for virulence of *Salmonella.* In 21 isolates *stn* gene was present, while *inv* A and *pef* A genes were present in 22 and 18 isolates respectively. In 15 isolates, all three virulent genes were present. The present study indicates that these isolates may be virulent and being a serious cause of health problems both in animals and humans.

Key words: River Ganges, water, *Salmonella*, pollution, virulence contributing genes.

Introduction

River Ganges has high religious and economic values in Indian subcontinent as its water is extensively used in agriculture, for drinking and other household purposes. It is considered as backbone of Indian agriculture but this river is becoming contaminated. The large quantities of pollutants such as untreated sewage material in large volume (Hamner et al., 2006) are disposed off in river Ganges that leads to accumulation of hazardous pathogens like *Shistosoma*, *Escherichia coli* O157:H7, *Shigella* and *Salmonella* (Hamner et al., 2007; Faruque et al., 2002; Tikko et al., 2001) and expose the surrounding population to these pathogens.

Salmonella is an important pathogenic organism which causes water-borne disease in humans and animals. Virulence of *Salmonella* is contributed by several genes, out of which few are very important like *inv* A, *stn* and *pef* A. Gene *inv* A was characterized by Galan et al. (1992) and was considered to be the first gene in an operon which is thought to trigger the internalization of *Salmonella* Typhimurium in cultured epithelial cells. Later it was found to be responsible for coding of proteins which are involved in invasion of *Salmonella* in reticulo-endothelial cells (Collago et al., 2006). Enterotoxic factor is encoded by *stn* gene which causes fluid accumulation and ileal secretion in intestinal mucosal glands and ultimately results into diarrhea (Mohan et al., 1995). Fimbrae of *Salmonella* is encoded by Gene *pef* A and helps in the attachment of *Salmonella* to intestinal mucosal cells (Murugkar et al., 2003). The presence of these three genes can be considered as marker for virulence of *Salmonella*.

In the present study, 500 water samples were collected from river Ganges. Fifty samples were collected from

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ten important religious and industrial stations like Gangotri, Uttarkashi, Rishikesh, Haridwar, Hastinapur, Garhmukteshwar, Narora, Kanpur, Varanasi and Allahabad. These samples were screened for presence of *Salmonella* by PCR and *Salmonella* was isolated from 44 positive samples. Out of 44 isolates, 24 were serotyped as *Salmonella* Typhimurium which has been considered pathogenic serovar of *Salmonella* and is a cause of disease in humans and animals. These 24 isolates of *Salmonella* Typhimurium were further screened for presence of three major virulence contributing genes *inv* A, *stn*, and *pef* A.

Material and Methods

Collection of Water Samples

Water samples were collected from ten stations of north India. These were Gangotri (30°49N/79°56E), Uttarkashi (30°43N/78°27E), Rishikesh (30°7N/78°19E), Haridwar (29°96N/78°16E), Hastinapur (29°10N/78°1E), Garmukeshwar (28°47N/78°05E), Narora (28°11N/78°23E), Kanpur (26°28N/80°21E), Allahabad (25°27N/81°51E) and Varanasi (25°20N/83°0E). Samples were collected from major bathing and cremation ghats. Each sample of 200 ml of water was collected in autoclaved bottles. Samples were brought to laboratory on ice within 12 hours of collection.

Filtration of Water Sample and Enrichment of *Salmonella*

Water samples were filtered with 0.22µ filter. Filter paper was transferred to 2 ml Luria bertinii broth (L.B. broth) (Hi-Media India) and incubated at 37°C overnight. One ml of culture in L.B. broth was transferred to nine ml of Tetrathionate broth (Hi-media India) and incubated at 41°C for 18 hrs. Loop full of culture was streaked on Brilliant Green Agar (Hi-media India) plate containing Novobiocin (50 µg/ml) and incubated at 37°C for 18 hours. Pink colonies were inoculated with 2 ml of L.B. broth for testing by PCR.

Salmonella Specific PCR

One ml of 18 hrs grown culture was centrifuged at 10,000 rpm for 10 minutes. Pellet was re-suspended in 100 µl of sterilized distilled water and kept in boiling water for 10 minutes followed by chilling on ice for 10 minutes. This heat-cold treatment caused lysis of the cells. Cell debris was removed by centrifugation at 5000 rpm for 10 minutes. Supernatant was collected and 5 µl of lysate was used for *Salmonella* specific PCR.

For *Salmonella* specific PCR primers (Cohen et al., 1994) targeting *his* J gene were used. Five µl of cell lysate was used as template DNA, 20 pmol of each primer, 200 µM of dNTPs and 1.5 mM $MgCl_2$ concentrations were used. All chemicals used in PCR were procured from MBI Fermentas, UK. PCR was conducted in Thermocycler (T-person MJ Research) with standard conditions. PCR program was as follows: initial denaturation at 94°C for five minutes followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for one minute. Each sample was amplified thrice along with a negative control to avoid any possibility of artifacts. Five µl of amplicon was mixed with 1 µl of 6× loading dye and loaded on 1% Agrose gel containing ethidium bromide (Sigma USA). Size of amplicon was measured by comparing with standard molecular weight marker (100 bp ladder, Bangalore Genei India).

Biochemical Characterization

Isolates showing positive results by PCR were tested by Hi *Salmonell*a identification kit (Hi-Media India). Each kit comprised 12 biochemical tests (Methyl Red, Vages prousker's Test, Urease, H_2S production, citrate utilization, lysine utilization, ONPG , lactose, arabinose, maltose, sorbitol and dulcitol fermentation test).

Serotyping of *Salmonella* Isolates

Isolates found positive in PCR and biochemical tests were sent to National *Salmonella* Research lab, Indian Veterinary Research Institute (Izatnagar-243122) for serotyping.

Detection of Virulence Contributing Genes

Detection of stn Gene

DNA was isolated from all isolates using C-TAB method. For detection of *stn* gene, the following primers were used (Murugkar et al., 2003).

Primer-1: 5′ TTG TGT CGC TAT CAG TGG CAA CC 3′

Primer-2: 5′ ATT CGT AAC CCG CTC TCG TCC 3′

25 µl of PCR reaction mixture containing 40 ng of template DNA, 200 µM of dNTPs, 20 pmole of each primer, 1U of Taq Polymerase with 1.5 mM final concentration of $MgCl_2$ was subjected to amplification of *stn* gene. PCR program was as follows: initial denaturation at 94°C for five minutes followed by 30 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for one minute. Each sample was amplified thrice along with a negative control to avoid any possibility of artifacts. Size of amplicon was measured by comparing with standard molecular weight marker (100 bp ladder, Bangalore Genei India).

Detection of inv A Gene

For detection of *inv A* gene following primers were used (Chiu and Ou, 1996).

Primer-1: 5′ ACA GTG CTC GTT TAC GAC CTG AAT 3′
Primer-2: 5′ AGA CGA CTG GTA CTG ATC GAT AAT 3′

Annealing temperature was kept at 56°C for 30 seconds; remaining conditions were similar to detection of *stn* gene by PCR.

Detection of *pef* A Gene

For detection of *pef* A gene following primers were used (Murugkar et al., 2003).

Primer-1: 5′ TGT TTC CGG GCT TGT GCT 3′
Primer-2: 5′ CAG GGC ATT TGC TGA TTC TTC 3′

Annealing temperature was kept at 56°C for 60 seconds; remaining conditions were similar to detection of *stn* gene by PCR.

Results and Discussion

Five hundred samples of water were screened for presence of *Salmonella* by PCR (Cohen et al., 1994). Out of 500 samples, 44 samples were found positive by *Salmonella* specific PCR. *Salmonella* was isolated from these samples and serotyped at National *Salmonella* Centre, IVRI Izatnagar (India). Out of 44 isolates, 24 were serotyped as *Salmonella* Typhimurium (Table 1). These were isolated from Haridwar, Hastinapur, Garmukteshwar and Narora. Most of these isolates were from Narora.

All the 24 isolates were screened for three virulence contributing genes: *stn*, *inv* A and *pef* A. In case of *stn* gene an amplicon of 617 bp was observed in 21 isolates, and in three isolates (G 36, G 52 and G 53) *stn* gene was not present (Figure 3, Table 1). *pef* A gene was found to be present in 18 isolates, as an amplicon of 700 bp was observed in these isolates (Figure 2). Out of 24 isolates, *inv* A gene was present in 22 isolates (Figure 1). Out of 24, all three genes were present in 15 isolates. These isolates have been tested for their multiple drug resistance and plasmid profiling. Most of these isolates have exhibited MDR against commonly used antibiotics and were possessing large plasmid which have been considered to play an important role in the virulence of *Salmonella* (Kaliyarasu et al., 2008). In India, untreated sewage material is generally disposed off in rivers, even in Ganges and Yamuna (Hammner et al., 2007; Gangwar and Joshi, 2004) and there are reports of isolation of pathogenically virulent *Salmonella* strains from these rivers (Lata et al, 2009; Shashi et al., 2009; Hammner et al., 2006; Singh et al., 2001; Ram et al., 2008).

Table 1: Distribution of virulence contributing genes in *Salmonella* Typhimurium

Name of Isolates	*Serovar*	*Place of Isolation*	*stn*	*pef A*	*Inv A*
G 1	*S.* Typhimurium	Haridwar	+	–	–
G 10	*S.* Typhimurium	Hastinapur	+	–	+
G 11	*S.* Typhimurium	Hastinapur	+	+	+
G 14	*S.* Typhimurium	Garmukteshwar	+	+	+
G 16	*S.* Typhimurium	Haridwar	+	+	+
G 20	*S.* Typhimurium	Garmukteshwar	+	+	+
G 21	*S.* Typhimurium	Garmukteshwar	+	+	+
G 23	*S.* Typhimurium	Narora	+	+	+
G 28	*S.* Typhimurium	Narora	+	–	+
G 29	*S.* Typhimurium	Narora	+	–	+
G 33	*S.* Typhimurium	Narora	+	+	+
G 35	*S.* Typhimurium	Narora	–	+	–
G 36	*S.*Typhimurium	Narora	+	+	+
G 37	*S.* Typhimurium	Narora	+	+	+
G 38	*S.* Typhimurium	Narora	+	–	+
G 40	*S.* Typhimurium	Narora	+	+	+
G 55	*S.* Typhimurium	Narora	+	–	+
G 44	*S.* Typhimurium	Narora	+	+	+
G 45	*S.* Typhimurium	Narora	+	+	+
G 46	*S.* Typhimurium	Narora	+	+	+
G 47	*S.* Typhimurium	Narora	–	+	+
G 50	*S.* Typhimurium	Narora	–	+	+
G 52	*S.* Typhimurium	Narora	+	+	+
G 53	*S.* Typhimurium	Narora	+	+	+

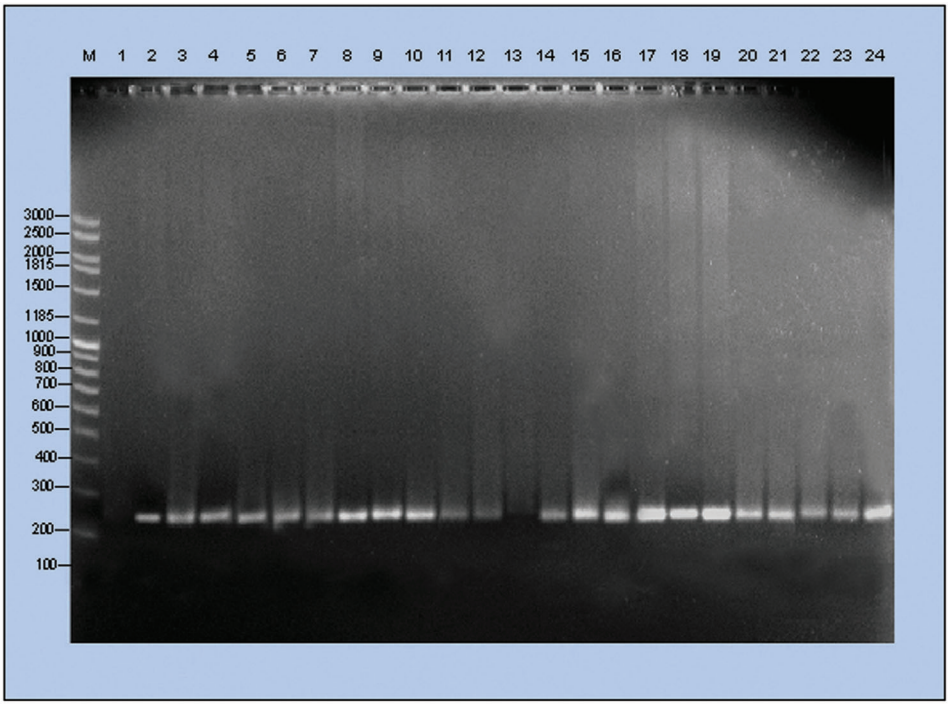

Figure 1: Inv Gene amplicon of Gangetic isolates of *Salmonella* Typhimurium
Lane M: Low Range DNA Rular Plus (Bangalore Genei, India)
Lane 1-24: *Salmonella* isolates (G1, G10, G11, G14, G16, G20, G21, G23, G28, G29, G33, G35, G36, G37, G38, G40, G44, G45, G46, G47, G50, G52, G53, G55)

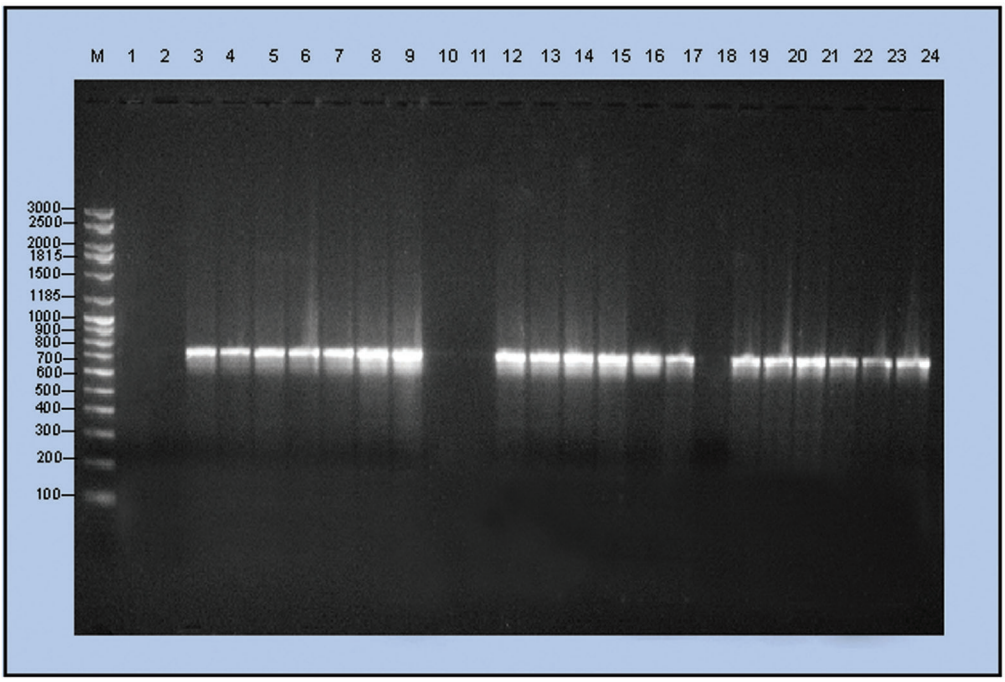

Figure 2: Pef Gene amplicon of Gangetic isolates of *Salmonella* Typhimurium
Lane M: Low Range DNA Rular Plus (Bangalore Genei, India)
Lane 1-24: *Salmonella* isolates (G1, G10, G11, G14, G16, G20, G21, G23, G28, G29, G33, G35, G36, G37, G38, G40, G44, G45, G46, G47, G50, G52, G53, G55)

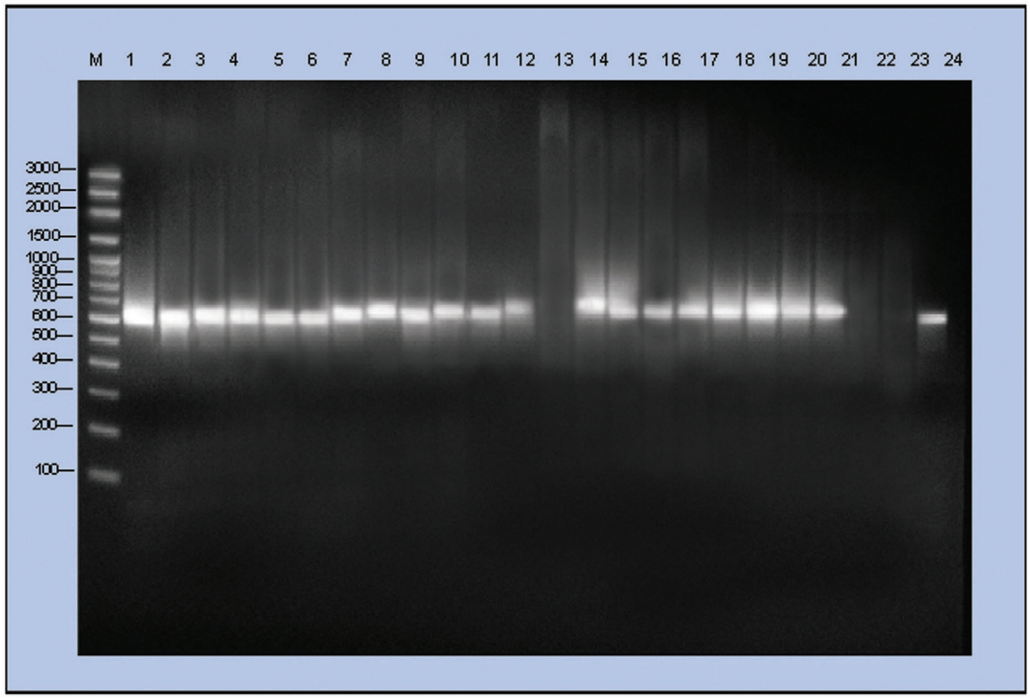

Figure 3: Stn Gene amplicon of Gangetic isolates of *Salmonella* Typhimurium
Lane M: Low Range DNA Rular (Bangalore Genei, India)
Lane 1-24: *Salmonella* isolates (G1, G10, G11, G14, G16, G20, G21, G23, G28, G29, G33, G35, G36, G37, G38, G40, G44, G45, G46, G47, G50, G52, G53, G55)

In our study, out of ten cities *Salmonella* Typhimurium was isolated from Ganges water from four cities. *Salmonella* Typhimurium is considered as dreaded pathogen (Singh et al., 2007) causing several life-theartening diseases such as endocarditis (Moreno et al., 2000), pneumonia (Genzen et al., 2008) and meningitis (Swe et al., 2008). Ganges water has high religious sentiments attached to it and it is not only used for bathing, household operations and agricultural operation but also used for drinking purposes both by humans and animals, thus a threat to animal and human population that thrive on it. The possible explanations of presence of this dreaded organism in Ganges water is disposal of untreated sewage material, unhygienic bathing conditions, disposal of dead bodies and industrial effluents.

Invasiveness of *Salmonella* is mediated by several chromosomal factors, *inv* A is considered as important virulence contributing gene and triggers the invasion of *Salmonella* in epithelial cells (Oliveria et al., 2003). PCR has been used for detection of *inv* A gene in virulent isolates (Kumar et al., 2009). In present study, *inv* A gene was found to be present in 22 isolates which indicates that these isolates are highly invasive. Another important virulence contributing factor is found to be responsible for production of enterotoxin and induces increased concentration of inositol phosphate and ultimately results into diarrhea and is coded by *stn* gene. Presence of *stn* gene is also considered as an important criteria for virulence determination and PCR has been used for its detection (Moore and Feist, 2007; Murugkar et al., 2003). In present study, *stn* gene was present in 21 isolates which further strengthens the virulence of these isolates. *pef* A gene mediates the attachment of *Salmonella* in epithelial cells of small intestinal. Presence of *pef* A gene in virulent isolates of *Salmonella* was demonstrated by PCR by Murugkar et al. (2003). In present study, it was present in 18 isolates. Out of 24, 15 isolates had all the three virulence contributing genes and is a strong indication that these isolates may be highly virulent.

On the basis of our findings and earlier available reports, we may conclude: (a) In Gangotri, Uttarkashi and Rishikesh, the Ganges is not polluted as from these stations we could not isolate *Salmonella* in any sample but in Haridwar it becomes polluted. (b) *Salmonella* Typhimurium was found to be most common serovar; it has high physiological adaptability and broad host range and most of the isolates were found to be pathogenic

and can be a cause of serious health problems both in humans and animals. (c) As reported globally, multiple drug resistance in *Salmonella* Typhimurium is also increasing in India, so the rational use of antibiotic is essentially required.

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References

Chiu, C.H. and J.T. Ou (1996). Rapid identification of *Salmonell*a serovars in feces by specific detection of virulence genes, *inv* A and *spv* C by enrichment broth Culture–multiplex PCR combination assay. *Journal of Clinical Microbiology*, **34:** 2619-2622.

Cohen, N.D., Neilbergs, H., Wallis, D.E., Simpson, R., Mcgruder, E.D. and B.M. Hargis (1994). Genus specific detection of *Salmonella* in equine feces by use of Polymerase chain reaction. *American Journal of Veterinary Research*, **55:** 1049-1054.

Collazo, C.M., Michael, K.Z. and J.E. Gatan (2006). Functional analysis of the *Salmonella* Typhimurium invasion genes *invl* and *invJ* and identification of target of the protein secretion apparatus encoded in the *inv* locus. *Molecular Microbiology*, **15:** 25-38.

Faruque, S.M., Khan, R., Kamruzzaman, M.Y., Shinji, Q., Ahmad, S., Azim, T., Nair, G.B., Tekeda, Y. and A.S. David (2002). Isolation of *Shigella dysenteriae* Type 1 and *S. flexneri* Strains from Surface Waters in Bangladesh: Comparative Molecular Analysis of Environmental *Shigella* Isolates versus Clinical Strains. *Applied and Environmental Microbiology*, **68:** 3908-3913.

Gangwar, K. and B.D. Joshi (2004). A preliminary study on solid waste generation at Har Ki Pauri, Haridwar, around the Ardh-Kumbh peri sacred bathing in the river Ganga in 2004. *Earth and Environmental Science*, **28:** 297-300.

Genzen, J.R., Towle, D.M. and J.D. Kravetz (2008) *Salmonella* Typhimurium pulmonary infection in an immunocompetent patient. *Conn Med*., **72:** 139-142.

Hamner, S., Broadway, S.C., Mishra, V.B., Tripathi, A., Mishra, R.K., Pulcini, E., Pyle, B.H. and E.F. Timothy (2007). Isolation of potentially pathogenic *E. coli* O157: H7 from Ganges River *Applied and Environmental Microbiology*, **73:** 2369- 2372.

Hamner, S., Tripathi, A., Mishra, R., Bouskill, N., Broadwy, S., Pyle, B. and T. Ford (2006). The role of water use patterns and sewage pollution in Incidences of waterborne /enteric disease along Ganga river in Varanasi, India. *International Journal of Environmental Health Research*, **16:** 113-132.

Kumar, R., Surendran, P.K. and N. Thampuran (2009). Detection and characterization of virulence factors in lactose positive and lactose negative *Salmonella* serovars isolated from seafood. *Food Control*, **20:** 376-380.

Lata, P., Ram, S., Agrawal, M. and R. Shanker (2009). Involvement of SPI-2-encoded SpiC in flagellum synthesis in *Salmonella enterica* serovar Typhimurium Enterococci in river Ganga surface waters. *BMC Microbiology*, **9:** 181-189.

Mohan, V.P., Sharma, K.B., Agrawal, D.S., Purnima, G. and P.K. Pillai (1995). Enterotoxin production and plasmid profiling of *Salmonella* Typhimurium isolated from the man. *Journal of Diarrheal Disease Research*, **13:** 166-171.

Moore, M. and M.D. Feist (2007). Real-time PCR method for *Salmonella* spp. targeting the *stn* gene. *Journal of Applied Microbiology*, **102:** 516-530.

Moreno, G., Moar, C., Roman, F., Perez, M.R., Lopez, D. and J.M. Letona **(**2000). *Salmonella* endocarditis presenting as cerebral hemorrhagez. *European Journal of Internal Medicine*, **11:** 96-97.

Murugkar, H.V., Rahman, H. and P.K. Dutta (2003). Distribution of virulence genes in *Salmonella* serovars isolated from man and animals. *Indian Journal of Medical Research*, **28:** 177-181.

Oliveira, S.D., Rodenbusch, C.R., Rocha, S.L. and C.W. Canal **(**2003). Evaluation of selective and non-selective enrichment PCR procedures for *Salmonella* detection. *Lett Appl Microbiol*., **36:** 217-221.

Ram, S., Vajpayee, P. and R. Shanker (2008). Contamination of portable water distribution system by multi antimicrobial resistant entrohaemorrhagic *E. coli*. *Environ Health Perspect*, **116:** 448-452.

Shashi, K., Batra, M., Umapati, V., Kumar, J. and M.K. Saxena (2009). Detection of Virulence in *Salmonella* Typhimurium isolated from Ganga Water. *International Journal of Applied Environmental Sciences*, **4:** 363-366.

Singh, D.V., Matte, M., Matte, G., Sunny, J., Sabeena, S., Shukla, S., Sanyal, S., Hog, A. and R. Colved (2001). Molecular analysis of *Vibrio cholerae* 01,0139, non- 01and non 0-139 strain clonally relationship between clinical and environmental isolates. *Applied Environmental Microbiology*, **67:** 901-921.

Singh, R., Shasany, A., Aggrawal, A., Sinha, S., Sisodia, B., Khanujia, S. and R. Mishra (2007). Low molecular weight

proteins of out membrane of *Salmonella* induced reactive arthritis revealed by proteomics. *Clin Exp. Immunology*, **148:** 486-493.

Swe, K., Nage, G., Van, M. and A. Hoosen (2008). S*almonella* Typhimurium meningitis in an adult patient with AIDS. *Journal of Clinical Pathology*, **61:** 138-139.

Tikko, A., Tripathi, A.K., Verma, S.C., Agrawal, N. and G. Nath (2001). Application of PCR fingerprinting techniques for identification and discrimination of *Salmonella* Isolates. *Current Science*, **80:** 1049-1052.

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