

Determination of Drug Resistance and Virulent Gene Signatures in Potable Water Isolates of *Escherichia coli* in Gwalior city

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Abstract:

Enterotoxigenic *Escherichia coli* (ETEC) is a causative agents of diarrhea. Humans and non humans can acquire infections primarily through consumption of contaminated water. In India, ETEC has been concerned as a foremost cause of diarrhea. The pathogen harbors *LT1* signature gene responsible for its virulence. The prevalence of drug resistance ETEC in water is of great concern. The present paper aims to isolate and characterize drug resistant virulent isolates of ETEC in potable water collected from different locations (n=6) of urban settings in Gwalior city. Further the samples were characterized using conventional culture methods and molecular methods. All identified ETEC isolates were subjected to antibiotic susceptibility testing. Our study indicates the existence of pathogenic drug-resistant isolates in potable water of Gwalior. Site 1 exhibited maximum intermediate isolates for antibiotics which is alarming and of great concern where as site 4 exhibited maximum resistance isolates of ETEC. This study may help in risk assessment posed by the infections due to persistence of antibiotic resistant bacteria in potable waters.

Keywords: Enterotoxigenic *E. coli*, Diarrhea, Drug resistance bacteria, Potable water, Virulence determinants.

INTRODUCTION:

Escherichia coli are one of the major habitats of intestinal tract in humans and animals [1]. Generally they are harmless, but certain pathogenic relative are common causes of infectious diarrhea and also for other enteric diseases [2]. They have been accepted as the potential indicators for microbial contamination in water. Contaminated water sources, surface and potable water are one of the main reservoirs of ETEC. ETEC strains cause diarrhea in infants and in adults by the production and release of enterotoxins, LT (heat labile enterotoxin) and ST (heat-stable enterotoxin). Children's under the age of five having poor immunity are most susceptible for ETEC infections [3, 4]. Persistence of drug resistance bacteria in environment and in clinical settings is always challenging. The exhaustive and over usage of drug to cure infections has leads the development of resistance mechanism in pathogenic strains. Ultimately these strains are prevalent and disseminated in water sources [5]. Multi drug resistant strains through poor and leaky water distribution system mixed with sewage pipelines reach the households in urban settings. Household waste as well as industrial effluents get discharged into the river. [6].

Antibiotics development has opened the avenue of possibilities for combating the infectious diseases [7, 8]. The emergence of antimicrobial resistance associated with plasmid is of great concern as it leads to the dissemination of resistant strains [9, 10, 11]. Fluoroquinolone is a group of antimicrobials which is used primarily for the therapeutics of intestinal infections. However, the emergence of Fluoroquinolone resistant bacteria has kept challenges before clinics and health personals. Therefore, the study on antibiotics resistance pattern both in terms of resistance and intermediate is of great concern for understanding their spread and emergence. Due to inadequate treatment plants the pathogenic bacteria harbouring antimicrobial resistant gene still persist in drinking water. This water supplied in households is used for both drinking and bathing purpose. The persistence of

antibiotic resistant microbes in potable water further pose challenges for the management of infectious diseases [9]. In urban environment, the water pipelines are often leaky and water gets contaminated with pathogenic microbes leading to possible waterborne disease outbreaks [12, 13]. The random use of antibiotics in clinics, easy accessibility of antibiotics without recommendation contributes to the appearance and diffusion of drug resistance in bacteria in environment [14]. In India and other developing countries, pathogen diagnostics based on antimicrobial agent resistance and virulence gene profiles of ETEC of potable water resources is not well established. Therefore, antibiotic resistance pattern of ETEC isolates recovered from potable waters will be helpful in future therapeutic advancements in humans and can stop the recycling of resistance bacteria in environment [15]. With the frequent use of antibiotics to kill Pathogenic *E. coli* in previous decades, the pathogen has evolved resistance mechanism to conflict against them. As a result, the drug-resistant ETEC strains are ubiquitous in environment resulting in high rates of morbidity and mortality [16]. The extensive use of antibiotics have generated and disseminated drug-resistant ETEC in the environment and potable water drinking system.

In the present work, we report the presence of antimicrobial-resistant of ETEC virulent signature gene in Potable water samples collected from different sites of Gwalior as a major city of Northern India.

MATERIALS AND METHODS:

Bacterial Isolates and strains:

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

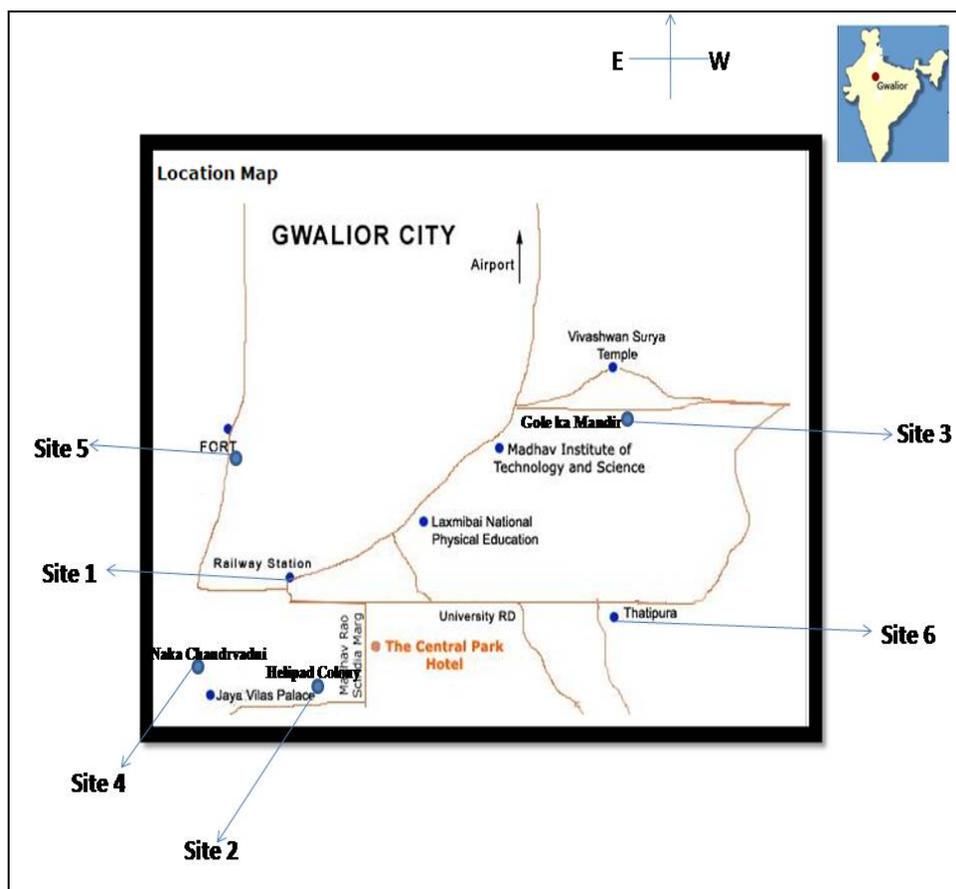


Figure 1. Locations of sampling sites of Gwalior city for collection of potable water

Description of sample locations:

Potable water samples were collected from the different sites located in the city. Treated water samples (water after passing through all purification stages from the treatment plants then pumping through the municipal taps into water tanker of Municipal Corporation for public consumption) were also collected. Samples (2 liters) were collected in sterilized bottles from six different sites in the Gwalior city. The water samples collected were processed immediately.

Primers:

Primers for *LT-1* gene were adopted from Ram et al. 2008 [17]. The specificity of adopted primers were further analyzed using NCBI-BLAST against the known microbial genome sequences to check the cross homology. Further, specificity of the primer set was validated using reference strain enterotoxigenic *Escherichia coli* MTCC 723.

Specificity Assay:

The inclusivity and exclusivity of the primer pair was checked using reference strains of *E. coli* and other genera. Different bacterial strains have been tested for specificity (Table 1) including one reference strain positive for *LT-1* gene (erotoxigenic *Escherichia coli* MTCC 723) and others negative for *LT-1* gene (*Escherichia coli* MTCC 1652, *Salmonella enterica* serovar Typhi MTCC 733, *Bacillus subtilis* MTCC 441 and *Bacillus megaterium* MTCC 453). DNA samples were isolated using boiling prep method followed by

precipitation and purification using ethanol and Sodium Acetate (3 M, pH 5.2). Further the PCR reaction was performed. The PCR reaction mixture in a final volume of 50 μ l consisted of dNTPs (0.2 mM), Taq DNA polymerase (0.75 unit), 10x reaction buffer (5 μ l), MgCl₂ (1.5 mM), primers (0.4 μ M, each) for *LT-1* gene and DNA template (5 μ l). The temperature profiling for PCR was as follows: initial denaturation at 95^oC for 4 min and then 30 cycles at 95^oC for 30 s, 55.8^oC for 45s, and 72^oC for 30s followed by final extension at 72^oC for 5 minutes.

Isolation and identification of Enterotoxigenic *E. coli*:

The samples from each site were filtered through membrane filter (cellulose nitrate filter 0.22 μ m pore size; Millipore, USA). The membrane filters immobilized with bacteria were aseptically removed and placed on culture media containing EMB agar (Hi-media, Mumbai) and incubated for further 12-15 h. The isolates confirmed as ETEC were maintained at -70^oC supplemented with 15% (vol. /vol.) glycerol.

Isolation of Genomic DNA:

DNA was extracted by boiling prep method. Briefly, *E. coli* isolates were incubated at 37^oC overnight. An aliquot (1ml) of the overnight culture was transferred to 2 ml tubes and centrifuged at 7000 rpm for 3 min. The supernatant was removed, and the pellet was resuspended by vortexing in 200 μ l of sterile double distilled water. The suspension was boiled for 30 min, centrifuged (7000 rpm, 2 min), and 250 μ l of the supernatant was collected and precipitation was done by using sodium acetate (0.3

M, pH 5.2) followed by ethanol. The precipitated DNA was washed thrice by 70% ethanol and resuspended in 100 µl TE (pH 8.0). The DNA quality of isolated DNA was checked in agarose gel electrophoresis.

Detection of virulence signatures of ETEC:

We selected 5 random *E. coli* isolates from each site for the presence of signature virulent gene *LT-1* (heat labile enterotoxin) using primers (Table 2) and thermal cyclic conditions. We used *E. coli* MTCC 723 as the positive control for *LT1* gene. The PCR reaction mixture and conditions used have been described earlier. All the assays were done in duplicate. Amplicons were analyzed on 1.7% agarose gel, visualized and recorded.

Antimicrobial drug susceptibility:

Five random isolates were selected for screening against antimicrobials from six identified sites. Total 30 isolates were used against eleven antibiotics from seven different classes as per CLSI guidelines. These are β -lactams {[Penicillinase resistant penicillins (anti-staphylococcal penicillins) Oxaciline 1 µg/disc], [(Amino benzyle penicillins) ampicillin, 10 µg/disc]}; Macrolides (Erythromycin 15 µg/disc); Aminoglycosides (amikacin, 10 µg/disc; tetracycline 30 µg/disc; streptomycin, 10 µg/disc); Phenicol (chloramphenicol, 10 µg/disc); Fluoroquinolones (ciprofloxacin, 5 µg/disc; norfloxacin, 10 µg/disc); Folate inhibitors (co-trimoxazole, 25 µg/disc); Lincosamides (Clindamycin 2 µg/disc); Each test was performed in triplicate. Data for antibiotic resistance of each isolate have been reported as resistant (R), isolates with reduced susceptibility (Intermediates) or sensitive (S), based on Clinical and Laboratory Standards Institute guidelines. It should be noted that *E. coli* strain DH5 α , (MTCC 1652) was used as negative control while *E. coli* strain ETEC, (MTCC 723) was used as positive control.

RESULTS AND DISCUSSION:

Bacterial Isolates & Strain:

In this study from 30 isolates of six different sites, four sites were positive for ETEC which were further characterized by the presence of virulent gene *LT1* using PCR.

In-silico specificity of primers:

The specificity of oligonucleotides was determined against the known microbial genomes by NCBI-BLAST. The BLAST analysis showed no cross homology in other genera or species [18].

Identification of virulent signatures of ETEC isolates:

Out of six sites four were positive for the presence of ETEC, harboring *LT-1*. The 150 bp amplicon were observed in these samples on gel electrophoresis after PCR. Results revealed that 66.66 % of ETEC strains isolated from the potable water exhibited virulent gene. Remaining isolates were negative for the presence of *LT1* gene (Table 1). Our interpretation on virulence markers specify that the potable water of Gwalior city is contaminated by ETEC isolates exhibiting *LT1* gene. Contaminated water and poor hygiene are the reasons for diarrheal diseases throughout the world [19]. Fifteen countries contribute three quarters of childhood deaths due

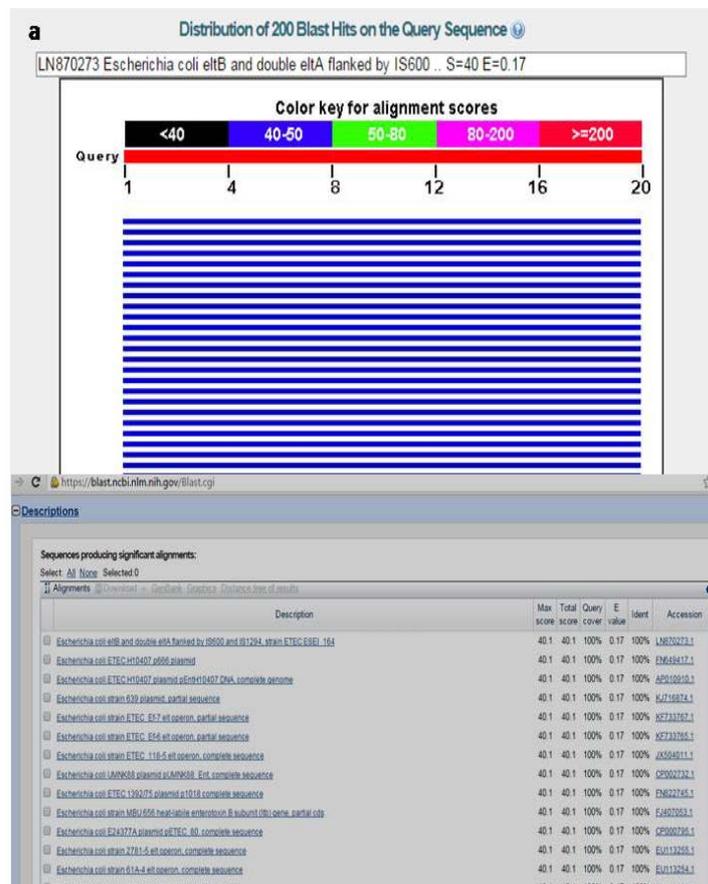
to diarrhea in children under five years of age worldwide out of which India ranks first [20]. In present study, we found potable water samples contaminated with strains of *E. coli*. The presence of ETEC in potable water suggests the possibility of contamination of water supplies [21]. Most of the strains 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 [22, 23]. Some of the isolates were intermediates for the resistance against antibiotics. These isolates can emergence as the resistant ones in near future [24, 25].

Table 1. Specificity Assay of *LT1* gene

S.No	Reference Strain	<i>LT-1</i> gene
1	ETEC MTCC 723	+
2	<i>Escherichia coli</i> MTCC 1652	-
3	<i>Salmonella</i> MTCC 733	-
4	<i>Bacillus Subtilis</i> MTCC 441	-
5	<i>Bacillus megaterium</i> MTCC 453	-

Table 2. Primer sequences of *LT-1* (heat labile enterotoxin) gene for the detection of ETEC

Gene	Primer Sequence (5-3)	Tm(°C)	Amplicon
<i>LT1</i>	GGCAGGCAAAAAGAGAAATGG	54.5	150 bp
	TTGGTCTCGGTCAGATATGTG	54.4	



Fig(a)

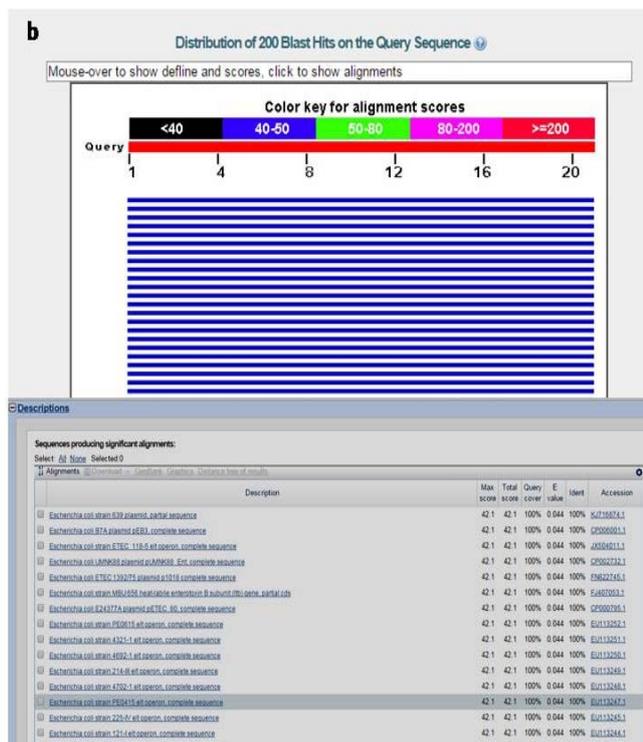


Figure 2. Graphical representation of NCBI BLAST for (a) forward primer and (b) reverse primer of LTI gene of E. coli

Antimicrobial Susceptibility:

β-lactams {(Penicillinase resistant penicillins(anti-staphylococcal penicillins) Oxaciline 1 µg/disc}, [(Amino benzyle penicillins) ampicillin, 10 µg/disc]}; Macrolides (Erythromycin 15 µg/disc); Aminoglycosides (amikacin, 10 µg/disc; tetracycline 30 µg/disc; streptomycin, 10 µg/disc); Phenicol (chloramphenicol, 10 µg/disc); Fluoroquinolones (ciprofloxacin, 5 µg/disc; norfloxacin, 10 µg/disc); Folate inhibitors (co-trimoxazole, 25 µg/disc); Lincosamides (Clindamycine 2 µg/disc) were used. ETEC is one of the important pathogens in water. Aquatic ecosystem could serve as a reservoir of antibiotic resistant pathogen through horizontal gene transfer mechanism. This study is on selected 20 drug resistant ETEC isolates from drinking water system of Gwalior city. These selected Drug resistant strains showed high resistance against Oxacilline, Ampicillin and Erythromycin (Table 3). A similar resistance pattern has also been observed in case of salmonella also which has also been isolated from potable water in Agarwal *et al* (2015). However, intermediate to Amikacine, Tetracycline, Streptomycin, Chloramphenicol, Co-trimoxazol, Clindamycine were observed among some isolates. Resistance to amoxicillin, nalidixic acid and tetracycline in EHEC isolates has also been observed in Ram *et al* (2011). Some of the strains were found to be sensitive against Ciprofloxacin and Norfloxin. The lesser number of antibiotics having bactericidal effect as compared to more antibiotics showing ineffectiveness against ETEC isolates is crucial and alarming.

In this study, five isolates were taken from each of the six different sites. The selected 30 isolates were checked for

the specificity of virulent gene, out of which 4 sites were found to be positive. It was found that site 4 showed high resistance pattern (41.81%) as compared to site 3 (36.36%), site 1 and site 2 (29.09%). Site 1 has high intermediate pattern (49.09%), whereas, site 2 (41.81%), site 4 (40%) and site 3 (34.54%) show lesser intermediate isolates. The sensitive isolates were observed in while, site2 and 3 shows similar sensitive pattern (29.09%), site 1 (21.81%) and site 4 (18.18%) has been observed against seven different classes of antibiotics (Figure 3).

Table 3. Mean zone of inhibition of ETEC against antibiotics

S. No.	Antibiotics	Mean Zone of Inhibition(mm)	Results (R/I/S)
1.	Ox ¹ Oxacilline	10 mm	Resistance
2.	Amp ¹⁰ Ampicillin	8 mm	Resistance
3.	E ¹⁵ Erythromycin	10 mm	Resistance
4.	AMK ¹⁰ Amikacine	17 mm	Intermediate
5.	TE ³⁰ Tetracycline	10 mm	Intermediate
6.	STR ¹⁰ Streptomycin	18 mm	Intermediate
7.	C ¹⁰ Chloramphenicol	18 mm	Intermediate
8.	CIP ⁵ Ciprofloxin	26 mm	Sensitive
9.	NOR ¹⁰ Norfloxin	22 mm	Sensitive
10.	Cot ²⁵ Co-trimoxazol	18 mm	Intermediate
11.	CD ² Clindamycine	16 mm	Intermediate

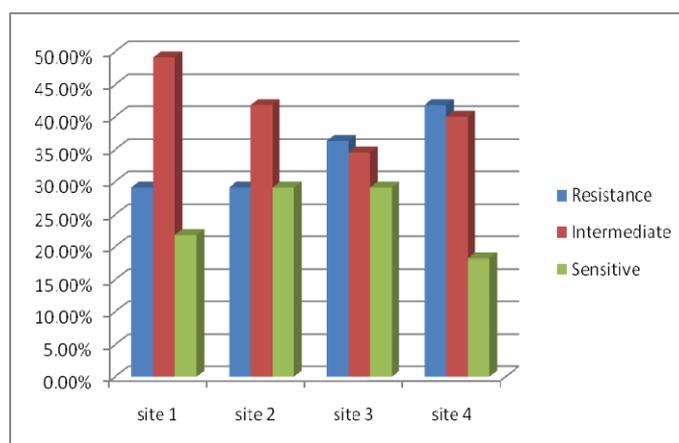


Figure 3: Percentage of Resistance, Intermediate (Moderate Resistance) and Sensitive against ETEC isolated from different sites.

CONCLUSION:

The study could be used for surveillance of potable water resources and development of risk assessment strategies for protection of public health. In summary, the occurrence of drug resistance in ETEC harbouring virulent genes in potable water signify that these environmental ETEC strains could significantly contribute to wide-scale transfer of virulent and resistance genes into aquatic and other healthy environments. This can lead to potential health hazard to human and animals which are dependent on water sources. This could play a major role in the epidemiology of antibiotic resistance in Gwalior the emergence of emerging and re-emerging diarrheagenic diseases.

ACKNOWLEDGEMENT:

We wish to express our sincere acknowledgement to Dr. Ashok Kumar Chauhan, President, RBEF parent organization of Amity University Madhya Pradesh (AUMP), Dr. Aseem Chauhan, Additional President, RBEF and chairman of Amity University Gwalior Campus, Lt. Gen. V.K. Sharma, AVSM (Retd.), Vice Chancellor of AUMP Gwalior Campus, for providing necessary facilities, their valuable support and encouragement throughout the work.

REFERENCES:

- Rosas, I., Salinas, E., Martinez, L., Calva, E., Cravioto, A., Eslava, C., Amabile-Cuevas CF., *Int. J. Hyg. Environ. Health.* 2006,209, 461-470.
- Clements, A., Young, J.C., Frankel, G., *Gut Microbes.* 2012, 3, 71-87.
- Tomar, R.S., and Jyoti, A., *Pharmanest.* 2015, 6, 2620-2624.
- Agarwal, M., Tomar, R.S., Jyoti, A., *Int. J. Multidisc. Current. Res.* 2014, 2, 351-358.
- Agarwal, M., Jyoti, A., Tomar, R.S., *Int. J. Pharm. & Biosci.* 2015, 6, 997-1004.
- Sharma, A., *Ind. J. Med. Micro. Bio.* 2011, 29, 91-92.
- Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, D., K.A., Handelsman J., *J. Nat Rev Microbio.* 2010, 8, 251-9.
- Cabello, F.C., *Environ Microbiol.* 2006, 8, 1137-44.
- Chen, B., Zheng, W., Yu, Y., Huang, W., Zheng, S., Zhang, Y., *et al. Appl. Environ. Microbiol.* 2011, 77, 148-155.
- Da.Silva, G. J., Mendoca, N., *Virulence.* 2012, 3, 18-28.
- Koczura, R., Mokracka, J., Jablonska, L., Gozdecka, E., Kubek, M., Kaznowski, A. *Sci. Total Environ.* 2012, 414, 680-685.
- Chávez, A., Maya, C., Gibson, R., and Jiménez, B. *Environ. Pollut.* 2011, 159, 1354-1462.
- Crane, J. K., S. S. Choudhari., T. M. Naeher., and M. E. Duffey. *Infect. Immun.* 2006, 74, 1505-1515.
- Lima. E., Guerr, R., *Chem. Cent. Jour.* 2013, 11, 1-7.
- Mahapatra, A., Padhi, N., Mahapatra, D., B, Mamta., S, Debasish., J, Swetlina., D, Debabrata., C, Nirupama., *Jou. Clinical Diag. Res.* 2015. 9, 9-11.
- Jyoti, A., Agarwal, M., Tomar, R.S., *Int. J. Res. App. Sci. & Eng. Tech.* 2015, 3, 1060-1064.
- Ram, S., Vajpayee, P., Shanker, R., *Environ. Sci. Technol.* 2008, 42, 4577-4582.
- Tomar, R.S., Jyoti, A., Mishra, R.K., Shrivastava, V., Kaushik, S., *Europ. Acad. Res.* 2014, 1, 5945-5958.
- Jyoti, A., Ram, S., Vajpayee, P., Singh, G., Dwivedi, PD., Jain, S.K., Shanker, R., *Sci. of Tot. Env.* 2010, 408, 1256-1263.
- Rathaur, V.K., Pathania, M., Jayara, A., Yadav, N., *J. Clin. Diag. Res.* 2014, 5, 01 - 05.
- Ram, S., Vajpayee, P., Dwivedi, P.D., Shanker, R., *Ecotoxicol. Environ. Saf.* 2011, 74, 551-557.
- Laam, Li., Mendis, N., Trigui, H., Oliver, J.D., Faucher, S.P., *Fron. Microbiol.* 2014, 5, 1-20.
- Ramamurthy, T., Ghosh, A., Gururaja, P.P., Shinoda, S., *Frontiers in Public Health.* 2014, 103, 1-9.
- Acharya, D., Malla, S., Bhatta, DR., Adhikari, N., Dumre, SP. *C Medic. Jour.* 2012, 37, 24-9.
- Abdullahi, B., Abdulfatai, K., Wartu, J.R., Mzungu, I., Muhammad, H.I.D., Abdulsalam, A.O., *African Jour. Microbiol. Res.* 2014, 8, 915-921.