A study on extended spectrum ß lactamase & metallo ß lactamase producing bacteria from drinking water sources in and around Kelambakkam

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Abstract

Introduction: Water is essential to life and many people do not have access to clean safe drinking water. Poor sanitation is the major cause for water contamination. Death due to infection like cholera, typhoid fever, bacillary dysentery are transmitted through contaminated water.

Aims & Objectives: To study the bacteriological profile of drinking water samples, to perform antimicrobial sensitivity testing of the isolates and genotypic identification of resistant gene.

Materials and Methodology: Drinking water sample were collected from in and around Kelambakkam for a period of 1year. Antimicrobial susceptibility testing was carried out for the isolates according to standard guidelines. ESBL producers were detected using double disc synergy test method. MBL producer were detected by combined disk test. Screening for *bla* TEM, *bla* VIM gene among the drug resistance isolates were done.

Result: Among 161 samples from various sources, most common isolates were Escherichia coli (n=41) 25.4%, followed by *Klebsiella spp.* Out of 41 isolates of *E.coli* were 23 and 10 were ESBL and MBL producer respectively. Among 10 isolates 8 were both ESBL and MBL producer, isolates positive for *bla* TEM gene & *bla* VIM gene were 5 & 2 respectively.

Keywords: *E.coli*, Drinking water, ESBL and MBL.

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Introduction

Water is essential to life but many people do not have access to clean safe drinking water and many die of waterborne bacterial infections.[1,3] Most important bacterial diseases transmitted through water-cholera, typhoid fever, bacillary dysentery and pathogenic Escherichia coli strains.[2]

Poor sanitation is the major cause for water contamination. Drinking water is a major source of microbial pathogen and considered to be one of the main reasons for increased mortality rates among children in developing countries Water.[3]

It was estimated that 88% of this burden is attributable to unsafe water supply, sanitation and hygiene, it is mostly concentrated in children in developing countries.[1,4] *Escherichia coli* is widely used as an indicator organism for the microbiological quality of water is also an important causative agent of diarrhea and other enteric diseases.[5]

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Antimicrobial resistance among enteropathogens, including *E. coli* has been reported to be increasing in recent years and sometimes leading to point-break situations where no antibiotic treatment options remain.[3]

Extended Spectrum β Lactamase (ESBL) producing bacteria have been reported increasingly worldwide they are often plasmid mediated and most of the enzymes are members of bla_{TEM} and bla_{SHV} families that have been described in many countries.[6] The bla_{TEM} was first reported in *E. coli* isolated from a patient named Temoniera in Greece. The name of the other beta-lactamase, bla_{SHV}, is due to sulfhydryl variable active site.[7]

The metallo-β-lactamase (MBL) show an extended substrate spectrum,including not only carbapenems, but also penicillins and the last- generation cephalosporins Spain.[8] Though they do not hydrolyze aztreonam, they are usually co-expressed with serine-β-lactamase that diminish the sensibility of the bacteria towards this compound.[9]

The metallo-β-lactamase (MBL) producers are usually co-expressed with serine-β-lactamase that diminish the susceptibility of the bacteria to penicillins, last generation of cephalosporins, even towards carbapenems group of drugs and they do not hydrolyze aztreonam.[8,9]

The cilinically relevant metallo-β-lactamases are encoded in mobile genetic elements and include VIMs (Verona Integron-encoded Metallo-β-lactamases) IMPs

(Imipenemase) and the more recently emerged NDMs (New Delhi Metallo-β-lactamase).[10]

Our aim was to study the bacteriological profile of drinking water samples, to perform antimicrobial sensitivity testing of the isolates and genotypic identification of resistant gene.

Materials and Methods

Water samples were collected for a period of 1 year (January 2015 to December 2015) in and around Kelambakkam. Samples were collected in sterile bottles of 150ml capacity from multiple sources. Care was taken to avoid contamination. Raw water and treated water from groundwater source and municipal water sources were collected. These sampling points were chosen for the study because they used for human consumption for drinking, recreational, agricultural and industrial purposes. So it is important to investigate the microbiological quality of water at these points.

Water samples were processed by (a) Multiple Tube Method[11] (b) Plate count method[11]

Multiple tube technique was done to enumerate the Most Probable Number (MPN) of Coliform bacteria. Measured volumes of water and dilutions of water are added to a series of tubes or bottles containing a liquid indicator growth medium. The media with one or more of the bacteria show growth and a characteristic color change. From the number, positive and negative reactions, the most probable number (MPN) of indicator organisms in the sample may be estimated by reference to statistical tables.[11]

The indicator medium used is MacConkey broth containing neutral red to indicate by its color change to yellow, the formation of acid from the lactose in the broth.

One bottle of 50 ml double strength MacConkey broth and 5 test tubes of 10ml double strength MacConkey broth and also 5 test tubes of 5ml single strength MacConkey broth with inverted Durham tubes for detection of gas were prepared. Then the test tubes were sterilized at 121^0C at 15 lbs.

Total coliform (presumptive coliform) count

1. Bottles and test tubes containing the 50ml and 10ml broth of indictor broth double strength and 5ml broth of single strength were taken with the inverted Durham tube, they were capped and sterilized.
2. The bottle containing samples of water were inverted rapidly several times to mix and distribute any deposit. A little of the water discarded aseptically, recapped and bottles were shaken up and down 25 times.
3. For waters of good quality, one 50ml volume and five 10ml volumes of the water samples were transferred aseptically into vessels containing corresponding 50ml and 10ml volumes of double strength medium.

For waters of doubtful quality, five 10ml volumes of the water was aseptically transferred into vessels containing the corresponding volumes of double – strength medium, and five 1ml volumes into vessels containing 5ml of single strength medium.

4. The seeded media were incubated aerobically at 37^0 C.
5. After 24h and 48h of incubation, the media were inspected and noted the number of cultures of each volume of water that show the production of acid (color change) and gas (a bubble large enough to fill the concavity at the top of the Durham tube) were noted. These acid and gas producing cultures are considered `presumptive positive` growths of coliform bacilli, e.g. *Escherichia coli, Klebsiella species or Citrobacter species*. Cultures not showing production of both acid and gas at 48h are considered negative.
6. By reference to tables of Most Probable Numbers(MPN) in respect of the combination of positive and negative results was observed.[11]

Plate count method

The plate count expresses the number of all colony forming bacteria in 1ml water. Separated plate cultures were incubated at $20\text{-}22^0$ C, grows mainly the natural saprophytes of soil and water, at 37^0C grows mainly parasitic bacteria derived from human and animal excretions. The true number of viable bacteria and yeasts in the water will be in excess of the counts on these plates, for many of the microorganisms occur in clumps and many fail to grow under the cultural conditions used.

1. Sterile yeast extract agar is melted and cooled and maintained at 50^0C until ready to pour plates.
2. A series of 10-fold dilutions of the water prepared aseptically by successively transferring 10ml volumes through a series of bottles holding 90ml sterile quarter strength Ringer`s solution as dilution.
3. Starting with the highest dilution of the sample and working towards the undiluted sample, exactly 1ml of the dilution or sample into each of four sterile petri dishes were aseptically added.
4. To each plate 15ml molten, cooled (50^0C) yeast extract agar were added immediately and the plates were gently rotated clockwise and then anticlockwise to mix the water with the medium. The agar was left undisturbed to set.
5. Two of the four plates of each dilution were incubated for 3days at $20\text{-}22^0$C and the other two for 24h+3h at 37^0C.
6. After incubation, colonies on each plate were counted. For counting the dilution plates that bear between 30 and 300 colonies were selected. Plate counts were obtained by multiplying the mean count by the dilution and it was expressed as the

number of colonies per ml water sample after 3days at 20-22^{0}C or 24h at 37^{0}C.

Less than 30 colonies on the plate from the undiluted sample, the count were reported as approximate only1. The plates from the highest dilution contain more than 300 colonies were reported as more than x colonies per ml, calculating x as the dilution factor multiplied by 300.

Enteric bacteria isolated were identified on the basis of their colony, morphological and biochemical properties according to standard guidelines.[11]

Antimicrobial susceptibility testing were done for the isolates according to standard guidelines. The antibiotics used were ampicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), piperacillin/tazobactam (100/10 µg), imipenem (10 µg), meropenem (10 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg), gentamicin (10 µg), amikacin (30 µg).[5]

E.coli isolates were predominant among the other isolates from drinking water drug resistant E.coli were screened for ESBL & MBL producers by **(**a) Double disk synergy test (Screening for ESBL detection) and (b) Combined disk test (Screening for metallo-beta-lactamase).

a. **Screening for ESBLs by double disk synergy test:** *Escherichia coli* that exhibited resistance to third generation cephalosporins were screened for ESBL producers as recommended by the (CLSI 2014)[5,12]. Cefotaxime 30µg was placed at a distance of 15mm edge to edge from a centrally placed of piperacillin/tazobactam. Plates were incubated at 35˚c for 18-20 hours and the pattern of zones of inhibition >5 increase zone diameter were positive for ESBL production.[12]

b. **Detection of Metallo-beta-lactamase production:[9,12]** Detection of MBL producers among the drug resistant *E.coli* were done by combined disk test according to standard guidelines.[12] Multidrug resistant *E.coli* were tested for metallo beta lactamases production by combined disk test. In this test, two imipenem discs (10 µg), one containing 10 µl of 0.1 M anhydrous EDTA (Himedia) were used. An increase in zone diameter of >5 mm around the imipenem-EDTA disc compared to that of the imipenem disc alone was considered positive for MBL production.[9]

PCR Amplification

bla*$_{TEM}$ gene and *bla*$_{VIM}$ gene detection among the drug resistant *Escherichia coli: After the phenotypic confirmation for ESBL and MBL production the *Escherichia coli* isolates were taken for genotypic identification. The presence of *bla*$_{TEM}$ gene and *bla*$_{VIM}$ gene detection among them were done by using the conventional PCR amplification (JASMN laboratory) with the appropriate primers purchased from Joyvel.

PCR Mixture

1. Primer -2µl
2. Template – 5µl
3. Master Mix – 12.5µl
4. Nucleas free solution – 5.5µl

Total Volume -25µl

Primers used for *bla*$_{TEM}$ and *bla*$_{VIM}$ gene[8,16]

Gene type		Primers
Gene for ESBL ***bla*$_{TEM}$**[16]	Forward	5**'**-AAAATTCTTGAAGACG -3'
	Reverse	5' -TTACCAATGCTTAATCA- 3**'**
Gene for MBL ***bla*$_{VIM}$**[8]	Forward	5'-GATGGTGTTTGGTCGCATA-3'
	Reverse	5'-CGAATGCGCAGCACCAG-3'

PCR Amplification procedure: 1µl of the stored DNA was added to the PCR mixture.

Initial Denaturation: 94˚C for 3minutes.
Denaturation: 94˚C for 1 minute.
Primer annealing: 55˚C for 1 minute
Heat stable extension: 72˚C for 1 minute 30 seconds.
Total number of cycles = 32.
Final Extension: 72˚C for 7 minutes.
Holding temperature: 40˚C

Steps

Initial denaturation: The complete Denaturation of the DNA template at the start of the PCR reaction is of key importance. The initial Denaturation was performed over an interval of 3min at 94˚C. The initial Denaturation was not longer than 3min at 94˚C, Taq polymerase were added into the initial reaction mixture.

Denaturation: Denaturation for 1min at 94-95˚C was sufficient; the PCR product synthesized in the first amplification cycle is significantly shorter than the template DNA and completely denatured under these conditions.

Primer annealing step: The optimum annealing temperature was 5˚C, lower than the melting temperature of primer-template DNA duplex. Incubation for 0.5-2min was sufficient.

Extension step: The extending step was performed at 70-72˚C. Extending time was 1min for the synthesis of PCR fragments.

Final extension step: After the last cycle, the samples were usually incubated at 72˚C for 7min to fill in the protruding ends of newly synthesized PCR products.

Number of cycles: The number of cycles depends on the number of the template DNA copies of the template DNA 32 cycles were performed.

Electrophoresis of DNA fragments

The process of electrophoresis was monitored by observing the migration of a visible dye (tracking dye) through the gel (bromophenol blue) that moves at the same speed as that of the double stranded DNA.

Visualization of DNA fragments

Dye Ethidium Bromide was added to agarose gel and isolation of bands was determined by the examination of the gel under the UV transilluminator.

Result

Total of 161 organisms were isolated from the drinking water samples collected in and around Kelambakkam district. Among 161 isolates, 41 were *E.coli* and 120 were other Gram negative bacteria. Isolation of Gram negative bacilli in water samples from Kelambakkam 51 (31.67%) and Vandaloor 24 (14.90%) were more than the bacterial isolates from Kovalam 21 (13.04%) Padur 26 (16.14%) & Thiriporur 39 (24.22%) area. [Table 1]

The drug resistant isolates in the water samples were 61 (37.88%). Among drug resistant Gram negative bacilli, the ESBL producers were 58 (39.02%) and MBL producing Gram negative bacilli were 21(13.04%). [Table 2]

Among the Gram negative bacilli isolated from water samples, 41 were *E.coli* and 34 (82.92%) isolates of *E.coli* were drug resistant. Among the *E.coli* isolates from the drinking water 23 were Extended Spectrum β lactamase producers and 10 were Metallo beta lactamases producer [Fig. 1].

Earlier studies from the clinical isolates of *E.coli* in our tertiary care hospital have documented the presence of bla $_{TEM}$ gene and bla $_{VIM}$ gene among drug resistant strains. The *E.coli* isolates from the water samples were phenotypically confirmed for both ESBL & MBL production and then taken for PCR Amplification. [Table 3] *E.coli* isolates were screened for bla_{TEM} gene & MBL positive isolates were screened for bla_{VIM} gene.[Fig. 2] Among the 8 *E.coli* which were positive phenotypically for ESBL and MBL production 2 (10.25%) were positive for bla_{TEM} gene and 5 (25.62%)(Fig. 3) were for bla_{VIM} gene positive. [Table 4]

Table 1: Bacterial Isolates from different areas in and around Kelambakkam

Isolates	Kelam Bakkam	Padur	Vandaloor	Kovalam	Thiru Porur
E.coli species	14	5	7	10	5
Klebsiella species	12	4	6	9	3
Psesudomonas species	3	4	2	4	3
Acinetobacter species	4	2	4	6	2

Table 2: Prevalance of ESBL and MBL producers in water samples

Isolates	ESBL (%)	MBL (%)
E.coli species	23(39.65%)	10(47.61%)
Klebsiella species	16(27.58%)	5(23.80%)
Psesudomonas species	8(13.79%)	2(9.52%)
Acinetobacter species	11(18.96%)	4(19.04%)

Table 3: Phenotypic detection of ESBL & MBL

Total number of *E.coli*	Double Disk Synergy Test For ESBL		Combined Disk Test for MBL		ESBL & MBL Both Producers	
41(25.46%)	+	-	+	-	+	-
	23 (56.09%)	18 (43.90%)	10 (24.39%)	31 (75.60%)	8 (19.51%)	33 (80.48%)

Table 4: E.coli with *bla*TEM gene & *bla*VIM gene

E.coli	ESBL	ESBL (*bla*TEM gene)		MBL	MBL (*bla*VIM gene)	
		(+)	(-)		(+)	(-)
No of isolates	(34.78%)	2(25%)	6(75%)	(80%)	(62.5%)	3(37.5%)

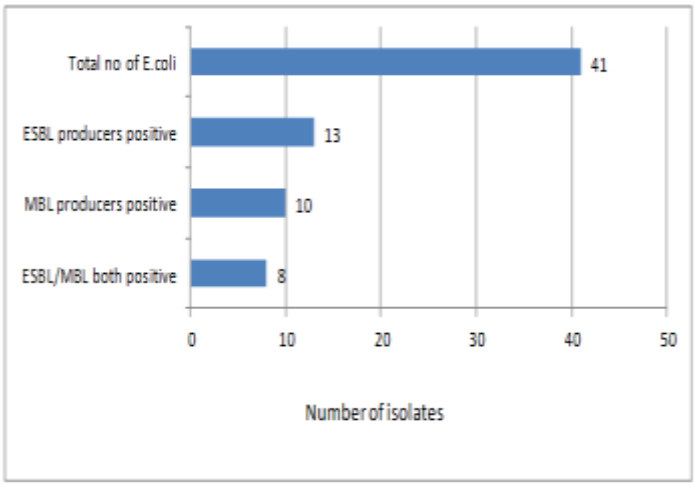

Fig. 1: ESBL & MBL producing strains of *E.coli*

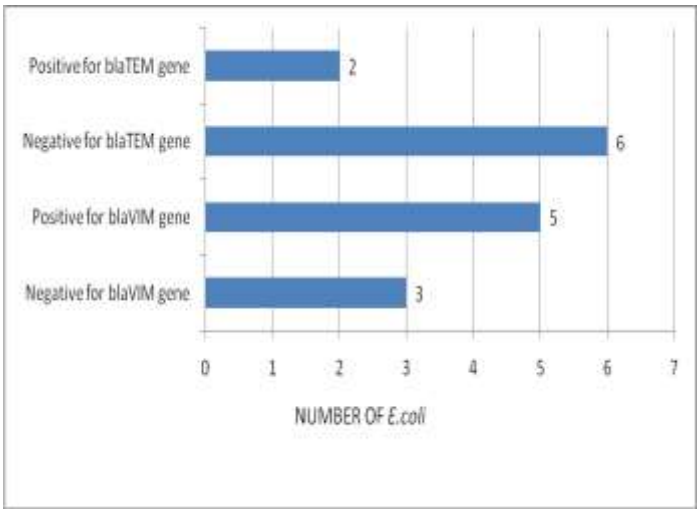

Fig. 2: Prevalence of *bla*VIM gene & *bla*TEM gene

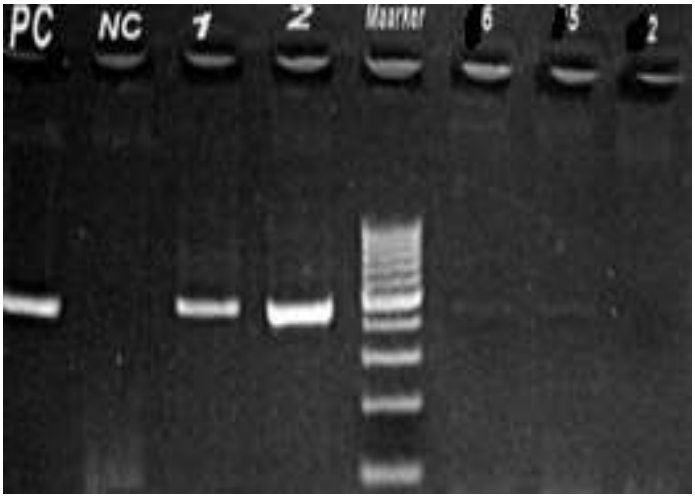

Fig. 3: Gel Picture *bla* TEM gene
Gel Picture: lane 1, 2- bla_{TEM} gene positive in E.coli
PC- Positive Control, **NC**- Negative Coli

Discussion

Analysis of water samples have documented the presence of microbial flora not only the Coliforms like *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus species*, *Klebsiella species* even *Staphylococcus species*, *Streptococcus species and Enterococcus species.*[15]

In our study 37.88% of the coliforms isolated from water samples were drug resistant and our study in concordance to increasing drug resistant bacteria from the drinking water samples by plate count method.[17]

The drug resistant bacteria in the water samples are more common because of the diversity of microbial flora in water, plasmid mediated transferable drug resistance. The antibiotic in aquatic environment determines as well as favors the drug resistant bacteria in water. Globally there are documentation of ESBL producing Enterobacteriaceae in water bodies in Portugal, Spain, Brazil, India.[14,18]

Gene transfer exceeds the limits of family, species and genera as they are horizontally transferable. The prevalence of drug resistant strain in water samples in near future responsible for transfer of drug resistance among the community acquired infection. More than 60% of Gram negative bacilli are able to transfer as well as exchange the plasmid (R plasmid) carrying resistance gene via horizontal gene transfer.[19]

In our study bla_{TEM} gene were present in 10.5% of the ESBL producers which was less to 56.7% bla_{TEM} gene positive *E.coli* isolated from the environmental samples by Junying Ma et al.,2012.[20]

In our study bla_{TEM} gene positive ESBL producers were documented and similar to study in river water for CTX-M-15 gene.[21] According Sharma J et al 2010 the bla_{TEM} gene in water sources were significant and in concordance with our study.[16] Fatemeh et al have documented the prevalence of bla_{TEM} gene among the ESBL producing *E.coli* and in concordance to our study.[6] Study of Manish K. Singh et al in 2015 have document the microbial contamination of packaged bulk drinking water sources in Karnataka in concordance to our study.[22]

MBL producers were documented with genomic profile among the clinical isolates. In our study bla_{VIM} gene were found in the *E.coli* isolated from water samples and the study of Fengxia Yang et al., 2016 documents the presence of bla_{IMP} gene among the carbapenemase gene.[19] Our study is similar to study of Schwatz et al., 2006 in documenting the bla_{VIM} gene in water samples.[23,24] We need to extend our study in analyzing more of drinking water samples, to do the genomic profile of them and to correlate with hospital strains.

Conclusion

Contamination of the drinking water is the common source of infection. Of which *Escherichia coli* has been found to be more predominant isolate than others. These situations are of serious concern in developing countries where enteropathogens are frequently encountered and cause life-threatening infections. So measures need to be taken and strictly followed to prevent water contamination and water borne disease as well as to control the persistence and spread of resistant bacteria.

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