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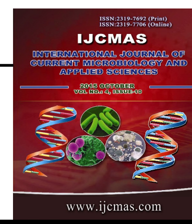

Original Research Article

Characterization of Extended Spectrum β-Lactamase Producing and Non-Producing *Escherichia coli* Isolated from Hospital and Fish Processing Plant Untreated Wastewaters

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Keywords

E coli, ESBL, *bla*$_{CTX-M}$, Antibiotic resistance genes, Hospital waste water, RAPD

ABSTRACT

This study aimed to detect and characterize Extended-Spectrum β-lactamase (ESBL) and non-ESBL producing *Escherichia coli* from untreated effluents let out from hospitals and fish processing plants in Mangalore, India. Isolates were phenotypically tested for ESBL production, resistance to ACCoT (ampicillin, chloramphenicol, co-trimoxazole, tetracyclines) and for the presence of their resistance encoded genes. ESBL-producing *E. coli* was seen to be dominant in hospital wastewaters (HWW) as compared to Fish processing wastewaters (FPWW). Among HWW ESBL positive strains 82% harbored the bla$_{CTX-M}$ gene, while bla$_{TEM}$ accounted for only 36%. These strains also showed resistance to ampicillin (100%), co-trimaxazole (59%), tetracycline (96%) and chloramphenicol(18%). Similarly non-ESBL HWW *E. coli* isolates showed resistance to ampicillin (92%), co-trimoxazole (64%), tetracycline (45%) and chloramphenicol (22%). The HWW ESBL and non-ESBL isolates were observed to be multi drug resistant with several encoding more than one gene determinant corresponding to their antibiotic resistance phenotype. RAPD-PCR for ESBL positive and negative *E coli* isolates from HWW and FPWW showed the existence of several genotypes among ESBL positive strains. No correlation existed between the ESBL phenotypes to antibiotic resistance genes harbored.

Introduction

The widespread use of antimicrobial agents in human, aquaculture and veterinary medicine has led to the emergence of multidrug resistant bacteria which is now a worldwide public health concern. Wastewaters discharged into the environment constitute a way for the introduction of antibiotic residues as well as antibiotic resistant bacteria into the environment (Harris *et al.,* 2012). *E. coli* a gram negative bacterium and an important microflora of the human and animal gastrointestinal tract has been frequently isolated from effluent waters fed into aquatic environments (Prado *et al.,* 2008; Chagas *et al.,* 2011). Beta-lactam antibiotics constitute the main therapeutic choice for treating human infections caused by *Enterobacteriaceae* bacteria. In recent years, extended-spectrum β-lactamase (ESBL) producing *E. coli* has gained recognition as a major clinical problem worldwide due to their increased resistance to most of the β-lactam antibiotics including penicillins, carbapenems and third generation cephalosporins (Banno *et al.,* 2004; Canton *et al.,* 2007). The increased resistance to β-lactams is due to the ability of bacteria to produce β-lactamase enzymes capable of hydrolyzing β-lactams, rendering the antibiotic inactive (Nordmann *et al.,* 2012).

Although many studies have reported the prevalence of ESBL producing *E. coli* in hospital settings (Pitout *et al.,* 2005; Korzeniewska and Harnisz, 2013), studies on its prevalence in hospital effluents and other non-clinical environments has been limited (Watkinson *et al.,* 2009).The aim of this study was to detect *E. coli* from wastewaters discharged from hospitals and fish processing plants and characterize them based on their ESBL production. The rationale for including fish processing wastewater was based on the common use of human intended drugs such as chloramphenicol, sulphonamide and tetracyclines in aquaculture practices, which has influenced the fecal coliforms to acquire resistance to the antibiotics used (Akinbowale *et al.,* 2007). ESBL and non-ESBL *E. coli* show simultaneous resistance to other non-β-lactam class of drugs (Bradford, 2001; Qin *et al.,* 2008) and therefore the *E. coli* strains in this study were tested for their antibiotic resistance to selected antibiotics and PCR detected further for the presence of implicated antibiotic resistant gene(s). The *E. coli* in this study was also genotyped by RAPD-PCR to determine their genetic relatedness and association to ESBL production and the antibiotic resistance genes harbored.

Materials and Methods

Bacterial strains

E. coli isolated from untreated wastewater let out from two hospitals (H1 and H2) and two fish processing plants (FP1 and FP2) were used in this study. The isolates were identified as *E. coli* by standard biochemical tests. Molecular identification of the strains using PCR primer was based on targeting a 146-bp fragment of the *uid*A gene (Table 1) by polymerase chain reaction (Asim *et al.,* 1991). Briefly, genomic DNA was extracted according to the method of Ausubel *et al.* (1995). The bacterial cell pellet was suspended in 567 µl of 1× TE buffer (10 mM Tris-Cl; 1 mM EDTA; pH 8.0), 30µl of 10% sodium dodecyl sulphate and 3 µl of proteinase K (20 mg/ml) and incubated at 45°C for 1 hour. After incubation, 100µl of 5M NaCl and 80µl of CTAB/NaCl solution were added and the mixture incubated for a further 10 min at 65°C. The solution was centrifuged at 10000 rpm for 10 min with an equal volume of chloroform/isoamyl

alcohol. The aqueous phase was transferred to a fresh tube and 0.6 volume of isopropanol added to precipitate DNA. The mixture was centrifuged and the pellet obtained was washed with 70% ethanol and re-centrifuged at 10,000 rpm for 5 min. The DNA pellet was vacuum dried and re-suspended in 100µl of sterile 1X TE buffer (pH 8.0). The DNA concentration and purity was estimated using a Nano Drop spectrophotometer (ND-1000, V3.3.0, Wilmington, DE, USA).

PCR was carried out in a programmable thermocycler (MJ Research, USA) using 30 µl reaction mixture containing 10X buffer (100mM of Tris- HCl, pH 8.3, 20mM 0f $MgCl_2$, 500mM of KCL and 0.1% gelatin) 200mM of deoxyribonucleotide triphosphate (dATP, dTTP, dGTP and dCTP), 10 picomoles of each primer and 1 U of *Taq* polymerase (Bangalore Genei, Bangalore), with 2.0 µl of template DNA. The optimized PCR programme consisted of an initial denaturation at 94°C for 5 min followed by 30 cycles with each cycle consisting of 94°C for 30 sec, Tm (annealing temperature) 60°C for 30 sec and extension for 72° C for 30 sec. The final extension was performed at 72°C for 10 min. The amplified products were resolved by 1.5 % (w/v) agarose gel electrophoresis.

Antibiotic susceptibility and ESBL production test

E.coli isolates were subjected to antibiotic susceptibility test on Mueller-Hinton agarby the Kirby-Bauer disk diffusion method (CLSI, 2012). Antibiotic discs (HI Media, Mumbai) tested were ampicillin (Amp, 10µg); cefotaxime (CTX, 30µg), ceftazidime, (CAZ, 30µg) chloramphenicol (C, 30µg), cotrimoxazole (CO, 25µg), tetracycline (TET, 30µg) and the resistance estimated as per CLSI guidelines (CLSI, 2012). *E coli* ATCC strain 25922 was used as a reference strain.

Strains resistant to cefotaxime and ceftazidime were tested further for the production of ESBL by double disc diffusion assay (DDDT). To a young lawn culture of the isolate on Muller-Hinton agar cephalosporin/clavulanate combination discs namely Ceftazidime (CAZ-30µg) and Ceftazidime + Clavulanic acid (CAC, 30/10µg), Cefotaxime (CTX, 30µg) and Cefotaxime + Clavulanic acid (CEC, 30/10µg) were placed. The plates were incubated for 24hrs at 37°C. An increase in zone diameter of equal or >5 mm around both CAC and CEC discs as compared to cefotaxime or ceftazidime tested alone were interpreted as positive for ESBL (Figure 1). *E. coli* ATCC 25922 was used as a standard strain.

Detection of antibiotic resistance genes

The *E. coli* strains were characterized for genes encoding β-lactamases ($bla_{CTX\text{-}M}$, $bla_{TEM\text{-}1}$), and for resistant genes that conferred resistance to chloramphenicol (*cat*1, *cat*2, *cat*3), sulfonamides (*sul*1, *sul*2, *sul*3) and tetracyclines (*tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G) by PCR assays. PCR reaction was carried out in a 30µl reaction mixture as described above using specific primers as mentioned in table 1. The amplicons were resolved in 1.5 % agarose gel, stained with ethidium bromide and visualized in a Gel Documentation system (Bio-Rad, USA).

RAPD-PCR assay

RAPD-PCR was performed initially using six randomly designed 10-mer oligonucleotide primers PM3 and PM5 (Tassanakajon *et al.,* 1997), CRA22, CRA26, OPA11 and OPA18 (Neilan 1995)

to evaluate and check for the repeatability of the fingerprints generated. PM5 was selected for further analysis as it yielded well resolved and better banding patterns. The RAPD-PCR was carried out in 30 µL reaction mixture consisting of 3µL of 10× PCR buffer (100 mM Tris-Cl pH 8.3, 20 mM $MgCl_2$, 500 mM KCl, 0.1% gelatin), 250 µM of each deoxynucleotide triphosphates, 50 pmol of primer, 1.5 U of *Taq* polymerase (Bangalore Genie, Bangalore, India). Standardized PCR thermo cycling conditions for the RAPD included initial denaturationat 95°C for 5 min and final delay at 72°C for 30s followed by 35 cycles, 94°C for 50s, and 36°C for 45sand 72°C for 30s. The amplification was carried out in a thermocycler (MJResearch, Watertown, USA) and the products resolved in 1.0% agarose gel, stained with ethidium bromide and the results photograph captured by Gel Documentation system (Bio-Rad, USA).

RAPD data analysis

Comparison of RAPD-PCR patterns generated for different gels was analysed using Gel compare II version 2.5 (Applied Maths, St Martens-Latem, Belgium). All visible electrophoretic bands were included in analysis. Similarities between profiles was based on Pearson's correlation coefficient and clustered using the unweighted pair group method with arithmetic mean (UPGMA). The similarity is expressed as percentage similarity and presented as a dendrogram. The numerical discriminatory index value was calculated (Hunter and Gatson 1988).

Results and Discussion

Characterization of β-lactamase-producing *E. coli*

The incidence of *E. coli* from FPWW in this study was lower than those isolated from HWW. *E. coli* isolated from hospital (n=150) and fish processing (n=32) wastewaters were screened for their antibiotic resistance patterns and ESBL production. A total of 114 (76%) and 12 (37.7%) *E. coli* isolated from HWW and FPWW respectively, showed resistance to one or more antibiotics tested. The remaining was sensitive to all the antibiotics tested. The antibiotic resistance strains were further characterized and grouped as ESBL and non-ESBL producing phenotypes. Of the 114 HWW isolates, 39 isolates were classified as ESBL producers by phenotypic assay. Analysis of the ESBL-encoding genes indicated that majority of the strains harbored CTX-M (82%) followed by bla_{TEM} (36%). Similarly for *E. coli* isolated from FPWW, only one isolate was positive for ESBL production and harbored the bla_{CTX-M} gene, while 2 among the 11 ESBL negative strains harbored bla_{TEM} gene (Table 2). All non-ESBL HWW isolates were negative for the presence of bla_{CTX-M} gene, while 19 (26%) isolates confirmed positive for the bla_{TEM} gene.

Antibiotic resistance and distribution of resistance genes

The HWW ESBL producing strains revealed complete resistance to ampicillin (100%), followed by resistance to cotrimaxazole (59%), tetracycline (46%) and chloramphenicol (18%).Afew of these strains also showed the simultaneous harboring of other antimicrobial resistance genes, the highest being for *sul* (41%), followed by *tet* (26%) and *cat* (3%) genes. The 74 non-ESBL HWW isolates showed resistance to ampicillin (92%), co-trimoxazole (64%), tetracyclines (45%) and chloramphenicol (22%). Among the non-ESBL 53%, 27% and 8% were observed to encode for *sul*, *tet* and *cat* genes

respectively. The encoding of more than one gene corresponding to an antibiotic resistant phenotype was also observed. For example, strains phenotypically resistant to co-trimoxazole were seen to encode either one or two of the *sul* genes, but not all gene determinants tested. This was also observed for strains resistant to tetracycline, wherein except for one strain which harbored four *tet* genes, the rest were seen to encode two, one or no *tet* genes (Table 2). The details of antibiotic resistance patterns and the distribution of antibiotic resistance genes for the *E. coli* included in this study are presented in table 2.

RAPD PCR

Twenty eight *E. coli* strains representative of ESBL positive and negative strains from both HWW and FPWW were used in RAPD analysis. RAPD analysis generated seven different banding patterns indicating the prevalence of different genotypes (Figure 2). The RAPD generated 6-12 bands with band sizes ranging from 0.3 kb to 1.2 kb. The number of bands generated for *E. coli* HWW isolates was observed to be higher in comparison to FPWW isolates (Figure 2). At a similarity of 70% all *E. coli* strains in this study grouped into eight clusters (C1-C8). All the non-ESBLs confirmed negative by phenotypic assay were seen to group into one large cluster C2 (Figure 2). However, the ESBL positive HWW isolates grouped themselves into seven clusters, with cluster C1 grouping 7 isolates, followed by cluster C5 (2 isolates) and the remaining (C3-C4, C6-C8) having a single isolate (Figure 2). Further, at 79% percent similarity value the ESBL producing HWW isolates were seen to be statistically discriminatory (DI=0.92), wherein the isolates in cluster C1 could be further sub grouped into C1a (4 isolates), C1b (2 isolates) and C1c (1 isolate). This indicates the prevalence of several genotypes among ESBL producing *E. coli* strains in hospital waster waters. The RAPD fingerprints generated showed no correlation between ESBL phenotype to antibiotic resistance genes harbored.

In this study the *E. coli* isolated from HPWW was higher as compared to FPWW. Almost all of the *E. coli* isolated from fish processing plant effluents were also observed to be sensitive to antibiotics tested. The low levels of resistance observed for FPWW *E. coli* in this study could be due to the low numbers identified or due to the non-use of these antibiotics following ban on the use of these antibiotics in aquaculture practice (Aquaculture News, 2003). However, the harboring of plasmid associated resistance genes such as bla_{TEM}, *sul* and *tet*seen in few of the FPWW isolates is a matter of concern. In contrast to FPWW, *E. coli* isolated from hospital effluents showed high resistance to antibiotics tested exhibiting several phenotypic resistance patterns. The higher multidrug resistant phenotypes could be due to the extensive usage of these drugs in hospitals for treating patients, which eventually gets released into hospital sewage. Majority (80%) of the β-lactamase producing ESBL phenotypes from the hospital sewage in this study encodedthe $bla_{CTX\text{-}M}$ gene, which is in accordance to observations made by other investigators (Amaya *et al.,* 2012; Galvin *et al.* 2010). The CTX-M type of ESBL strains has been responsible for outbreaks in hospitals (Rezai *et al.,* 2015) and environments (Pitout *et al.,* 2005; Rossolini *et. al*., 2008) throughout the world. A few strains positive for the $bla_{CTM\text{-}M}$gene also co-harbored the bla_{TEM} gene. The presence of more than one *bla*-gene has been previously documented for *E. coli* isolated from sewage (Korzeniewska and Harnisz, 2013, Galvin *et al.,* 2010) and the reason suggested for the high-levels of β-lactamase resistance (Kiratisin *et al.,* 2008).

Table.1 Primers used in this study

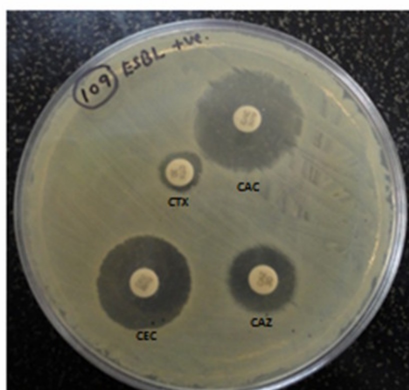
Primer	Primer sequence (5'-3')	Product size (bp)	Reference
bla_{CTX-M}	F:ACGTTAAACACCGCCATTCC R:TCGGTGACGATTTTAGCCGC	356	Colomer-Lluch Marta *et al.*, 2011
bla_{TEM}	F:CTCACCCAGAAACGCTGGTG R:ATCCGCCTCCATCCAGTCTA	569	Colomer-Lluch Marta *et al.*, 2011
cat1	F:AACCAGACCGTTCAGCTGGAT R:CCTGCCACTCATCGCAGTAC	549	Ma *et al.*, 2007
cat2	F:AACGGCATGATGAACCTGAA R:ATCCCAATGGCATCGTAAAG	547	Ma *et al.*, 2007
cat3	F:ATCGGCATCGGTTACCATGT R:ATCCCCTTCTTGCTGATATT	531	Ma *et al.*, 2007
sul 1	F:TTTCCTGACCCTGCGCTCTAT R:GTGCGGACGTAGTCAGCGCCA	425	Ma *et al.*, 2007
sul 2	R:CCTGTTTCGTCCGACACAGA R:GAAGCGCAGCCGCAATTCAT	435	Ma *et al.*, 2007
sul 3	F:ATGAGCAAGATTTTTGGAATCGT R:CTAACCTAGGGCTTTGGATATTT	792	Ma *et al.*, 2007
tetA	F:TTGGCATTCTGCATTCACTC R:GTATAGCTTGCCGGAAGTCG	494	Ma *et al.*, 2007
tetB	F:CAGTGCTGTTGTTGTCATTAA R:GCTTGGAATACTGAGTGTTAA	571	Ma *et al.*, 2007
tetC	F:CTTGAGAGCCTTCAACCCAG R:ATGGTCGTCATCTACCTGCC	418	Ma *et al.*, 2007
tetD	F:GCAAACCATTACGGCATTCT R:GATAAGCTGCGCGGTAAAAA	546	Ma *et al.*, 2007
tetE	F:TATTAACGGGCTGGCATTTC R:AGCTGTCAGGTGGGTCAAAC	544	Ma *et al.*, 2007
tetG	F:GCTCGGTGGTATCTCTGCTC R:CAAAGCCCCTTGCTTGTTAC	550	Ma *et al.*, 2007
PM5	CGACGCCCTG	-	Tassanakajon *et al.*, 1997
*uid*A	F:AAAACGGCAAGAAAAAGCAG R:ACGCGTGGTTACAGTCTTGCG	146	Asim *et al.*, 1991

Table.2 Antibiotic resistance profile and the distribution of resistance genes among ESBL and non-ESBL producing *E. coli*

Antibiotic resistance profile	**No. of isolates PCR positive for the antibiotic resistant gene corresponding to**				
	ESBL (bla_{CTX-M})	(bla_{TEM})	**Chloramphenicol** (*cat1, cat2, cat3*)	**Cotrimoxazole** (*sul1, sul2, sul3*)	**Tetracycline** (*tetA,tetB, tetC, tetD, tetE*)
HWW ESBL+veE.coli					
A, (*n*=9)	+ (8)	+ (4)	-	-	-
A,C (*n*=2)	+ (1)	-	-	-	-
A,C,Co (*n*=2)	+ (2)	+ (1)	-	*sul2*(1)	-
A,Co (*n*=8)	+ (6)	+ (4)	-	*sul1*(1); *sul2*(1); *sul3*(1); *sul1,2*(1); *sul2,3*(1)*	-
A,Co,T (*n*=10)	+ (9)	+ (3)	-	*sul1*(1); *sul3*(1); *sul1*,2(4); *sul1,3*(1)	*tetE*(1); *tetC*(2); *tetA,E* (1); *tetA,D*(1)
A,C,Co,T (*n*=3)	+ (1)	+ (1)	*cat1*(1)	*sul3*(1); *sul1,2,3*(2)	*tetA*(1); *tetA,C*(1)
A,T (*n*=5)	+ (5)	+ (1)	-	-	*tetA*(1); *tetD*(1); *tetA,D*(1)
HWW ESBL-veE.coli					
A (*n*=11)	-	-	-	-	-
C (*n*=1)	-	-	-	-	-
Co (*n=3*)	-	-	-	*sul1*(1); *sul1,2*(1)	-
A,C (*n*=3)	-	+ (2)	*cat1,2*(2)		
A, Co(*n*=18)	-	+ (6)	-	*sul1,2*(3); *sul1*(6); *sul2*(2); *sul2,3*(1); *sul1,3*(1); *sul3*(2)	-
A,C, Co (*n*=5)	-	+ (2)	*cat2*(1)	*sul1*(1); *sul2*(3); *sul1,2*(1)	-
A, C, Co, T (*n*=6)	-	+ (2)	*cat1,2*(2); *cat2*(1)	*sul1*(3); *sul2*(2)	*tetA*(1); *tetD*(1); *tet B,D*(1)
A, C, T (*n*=1)	-	-	-	-	-
A,Co,T (*n*=15)	-	+ (3)	-	*sul1*(7); *sul1,2*(4); *sul2*(1)	*tetB,E*(1); *tetA*(2); *tetB*(1);*tetD*(1); *tetB,C,D,E*(1); *tetC(1)*
A, T (*n*=9)	-	+ (4)	-	-	*tetE*(1); *tetC*(1); *tetA,B*(1);*tetA*(4);*tetA,E*(1)
T (*n*=2)	-	-	-	-	*tetA*(2)
FPWW ESBL +veE.coli					
CAZ, CTX, T(*n*=1)	+ (1)	-	-	-	-
FPWW ESBL -veE.coli					
A (*n*=3)	-	-	-	-	-
T (*n*=3)	-	-	-	-	-
A, T (*n*=3)	-	+ (2)	-	-	*tet*D(1)
A, Co (*n*=2)	-	-	-	*sul*1(1)	-

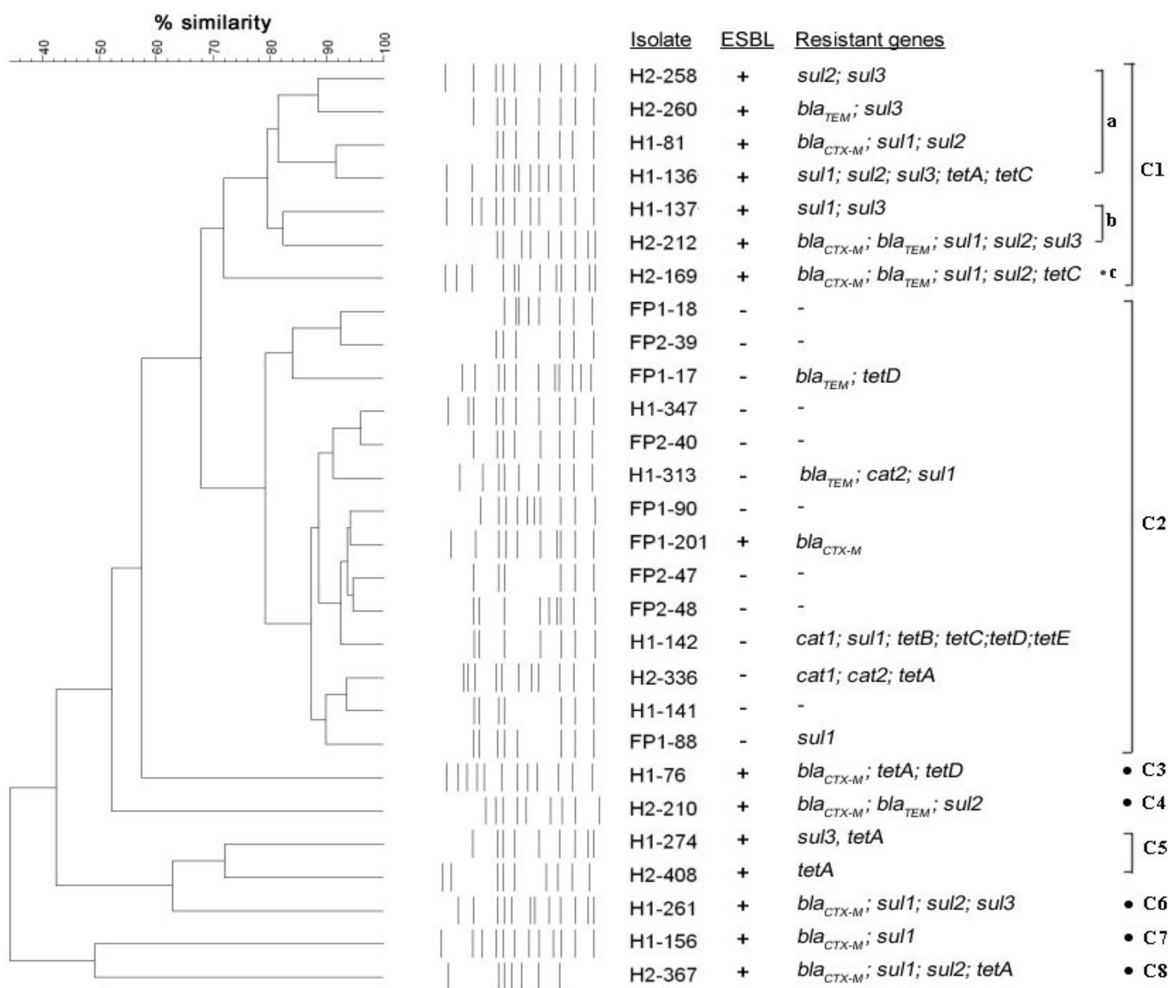
A, ampicillin; C, chloramphenicol; Co, co-trimoxazole; T, tetracycline; HWW: Hospital wastewater; FPWW: Fish processing plant wastewater, The HWW ESBL isolates were resistant to ceftazidime and cefotaxime. 'n' indicates the number of isolates and no. in brackets indicates the no of isolates positive for the gene; *presence of more than one gene coding for the same resistant antibiotic is separated by a comma

Figure.1ESBL detection by double disk diffusion test



CAZ: ceftazidime; CAC: ceftazidime + clavulanic acid; CTX: cefotaxime; CEC: cefotaxime + clavulanic acid

Figure.2 Dendrogram generated based on RAPD PCR profiles for ESBL and non-ESBL E.coli isolates. H1 and H2 represents hospital wastewater isolates and FP1 and FP2 fish processing wastewater isolates. C1-C8 are clusters generated at 70% similarity



The TEM gene has been associated with 90% of ampicillin resistance in *E. coli* (Livermore, 1995). In this study, a low association of 36% and 26% was observed for HWW ESBLs and non-ESBL respectively. A small percentage of the *E. coli* positive for the production of β-lactamase but negative for the presence of the *bla* genes could be harboring other variant genes such as SHV, OXA, PER types implicated in ESBL production which was not included in this study.

Several of the *E. coli* strains in this study were observed to simultaneously harbor antibiotic genes associated with non-β lactam class of antibiotics (Table 2). It was interesting to note that many of the ESBL and non-ESBL isolates that displayed resistance to co-trimoxazole and tetracycline showed the presence of multiple determinants of *sul* and *tet* genes respectively.

Genotyping of *E. coli* by RAPD-PCR showed that there exists a genetically diverse and heterogeneous group among ESBL and non-ESBL producers in hospital wastewaters, which maybe a reflection of genotypes circulating within the hospitals. The prevalence of several genotypes in hospital waste waters thus emphasizes the need for appropriate control measures to be taken before letting out untreated water into the environment. Further, the isolates are multidrug resistant and carry plasmid mediated antibiotic resistance genes which could transfer these genes to other non-resistant bacterial strains through exchange of genetic material (Rahube, 2010). Therefore molecular detection and identification of ESBL producing *E. coli* in hospital untreated waste water becomes important for implementation of appropriate control measures. Our studies with RAPD show although RAPD-PCR cannot be used in typing strains based on β-lactam or non β-lactams genes they carry, this tool could be used as a technique for epidemiologically discriminating ESBL producers from non-ESBL producing *E. coli* strains.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Reference

Akinbowale, O.L., Peng, H., Barton, M.D., 2007. Class 1 integron mediates antibiotic resistance in *Aeromonas spp*. from rainbow trout farms in Australia*. Int. J. Antimicrob. Agents.,* 29: S113.

Amaya, E., Reyes, D., Paniagua, M., Calderon, S., Rashid, M.U., Colque, P., Kuhn, I., Mollby, R., Weintraub, A., Nord, C.E. 2012. Antibiotic resistance patterns of Escherichia coli isolates from different aquatic environmental sources in Leon, Nicaragua. *Clin. Microbiol. Infect.,* 18: E347–E354.

Aquaculture News, 2003. List of antibiotics banned in India, Marine Products Exports Authority of India-Cochin. Pp. 2–3.

Asim, K.B., Joseph, L.D., Lawrence, H., Ronald, M.A. 1991. Detection of *Escherichia coli* and *Shigella spp* in

water by using the polymerase chain reaction and gene probes for uid. *Appl. Environ. Microbiol.,* 57: 1013–17.

Ausubel, F.M. 1995. Short protocols in molecular biology, 3rd edn. Wiley, New York, USA.

Banno, R.J., Navarro, M.D., Romero, L., Martinez, L.M., Munian, M.A., Perea, E.J., Cano, R.P., Passual, A. 2004. Epidemiology and clinical features of infections caused by extended-spectrum β-lactamase-producing *Escherichia coli* in non-hospitalized patients. *J. Clin. Microbiol.,* 42: 1089–94.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M. 1966. Antibiotic susceptibility testing by standardized single disc method. *Am. J. Clin. Pathol.,* 45: 493–96.

Bradford, P.A. 2001. Extended spectrum of beta lactamases in 21st century: characterization, epidemiology and detection of this important threat. *Clin. Microbiol. Rev.,* 14: 1933–51.

Canton, R., Valverde, A., Novais, A., Baquero, F., Coque, T. 2007. Evolution and current situation of ESBL. *Enferm. Infec. Microbiol. Clin.,* 25(S2): 2–10.

Chagas, T.P.G., Seki, L.M., Cury, J.C., Oliveira, J.A.L., Davila, A.M.R., Silva, D.M., Asensi, M.D. 2011. Multiresistance, β-lactamase-encoding genes and bacterial diversity in hospital wastewater in Rio de Janeiro, Brazil. *J. Appl. Microbiol.,* 111: 572–81.

Clinical and Laboratory Standard Institute. Performance standards for antimicrobial susceptibility testing, 2012. 22nd informational supplement, CLSI, Wayne. 32: M100–S22.

Colomer-lluch, M., Jofre, J., Muniesa, M., 2011. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS ONE.,* 6: e17549.

Galvin, S., Boyle, F., Paul Hickey, P., Vellinga, A., Morris, D., Cormican, M. 2010. Enumeration and characterization of antimicrobial-resistant Escherichia coli Bacteria in effluent from municipal, hospital, and secondary treatment facility sources. *Appl. Environ. Microbiol.,* 76: 4772–79.

Harris, S.J., Cormican, M., Cummins, E. 2012. Antimicrobial residues and antimicrobial-resistant bacteria: impact on the microbial environment and risk to human health-a review. *Hum. Ecol. Risk Assess.,* 18: 767–809.

Hunter, R.P., Gaston, A.M. 1988. Numerical index of the discriminatory ability of typing systems: an application of simpsons index of diversity. *J. Clin. Microbiol.,* 6: 2465–66.

Kiratisin, P., Apisarnthanarak, A., Laesripa, C., Saifon, P. 2008. Molecular characterization and epidemiology of extended-spectrum-β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates causing health care-associated infection in Thailand, where the CTX-M family is endemic. *Antimicrob. Agents. Chemother.,* 52: 2818–24.

Korzeniewska, E., Harnisz, M. 2013. Beta –lactamase- producing Enterobacteriaceae in hospital effluents. *J. Environ. Management,* 123: 1–7.

Livermore, D.M. 1995. Beta-lactamases in laboratory and clinical resistance. *Clin. Microb. Rev.,* 8: 557–84.

Ma, M., Wang, H.I., Yu, Y., Yong., Zhang, D., Liu, S. 2007. Detection of antimicrobial resistance genes of

pathogenic *Salmonella* from swine with DNA microarray. *J. Vet. Diagn. Invest.,* 19: 161–167.

Neilan, B.A. 1995. Identification and phylogenetic analysis of toxigenic cyanobacteria by multiplex randomly amplified polymorphic DNA PCR. *Appl. Environ. Microbiol.,* 61: 2286–91.

Nordmann, P., Dortet, L., Poirel, L. 2012. Carbapenem resistance in Enterobacteriaceae: here is the storm. *Trends Mol. Med.,* 18: 263–72.

Pitout, J.D.D., Nordmann, P., Laupland, K.B., Poirel, L. 2005. Emergence of Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) in the community. *J. Antimicrobial. Chem.,* 56: 52–59.

Prado, T.M., Pereira, W.C., Silva, D.M., Seki, L.M., Carvalho, A.P.D.A., Asensi, M.D. 2008. Detection of extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* in effluents and sludge of a hospital sewage treatment plant. *Lett. Appl. Microbiol.,* 46: 136–41.

Qin, X., Zerr, D.M., Weissman, S.J., Englund, J.A., Denno, D.M., Klein, E.J., Tarr, P.I., Kwong Stapp., J.R., Tulloch, L.G., Galanakis, E. 2008. Prevalence and mechanisms of broad – spectrum beta-lactam resistance in Enterobacteriaceae: a children's hospital experience. *Antimicrob. Agents Chemother.,* 52: 3909–14.

Rahube, T.O., Christopher, K.Y. 2010. Antibiotic resistance plasmids in wastewater treatment plants and their possible dissemination into the environment. *Afr. J. Biotechnol.,* 9: 9183–90.

Rezai, M.S., Salehifar, E., Rafiei, A., Langaee, T., Rafito, M., Shafahi, K., Eslami, G. 2015. Characterization of multidrug resistant extended-spectrumbeta-lactamase-producing *Escherichia coli* among uropathogens of pediatrics in North of Iran. *Biomed. Res.,* Pp. 1–7.

Rossolini, G.H., D'Andrea, M.M., Mugnioli, C. 2008. The spread of CTX-M type extended- spectrum beta-lactaamses. *Clin. Microbiol. Infect.,* 14: 33–41.

Tassanakajon, A., Pongsomboon, S., Rimphanitchayakil, V., Jarayabhaud, P., Boonsaeug, V. 1997. Random amplified polymorphic DNA (RAPD) markers for determination of genetic variation in wild population of the black tiger prawn (Penaeusmonodon) in Thailand. *Mol. Marine Biol. Biotechnol.,* 6: 110–15.

Watkinson, A.J., Murby, E.J., Kolpin, D.W., Costanzo, S.D. 2009. The occurrence of antibiotics in an urban watershed: from waste water to drinking water. *Sci. Total Environ.,* 407: 2711–23.