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Research Article

Antibiotic resistant patterns and plasmid profile of *E.coli* strains isolated from clinical samples of Cuddalore district, Tamilnadu

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Abstract

The present study deals with 175 strains isolated from different hospitals located at Chidambaram, Parangipettai and Cuddalore. Ampicillin (AMP) - 25µg, Cefuroxime (CXM) - 30µg, Amoxcillin (AMC) - 30µg, Cefpodoxime (CPD) - 10µg, Cephalexin (CN) - 30µg, Doxycycline (DO) - 30µg, Levofloxacin (LE) - 5µg, Gentamicin (GEN) - 10µg, Ciprofloxacin (CF) - 5µg, Norfloxacin (NX) - 10µg and Ofloxin (OF) - 5µg were the eleven antibiotics tested, to which highest resistance was noted towards ampicillin and least resistance was towards Ampicillin (100%). Around 8.5% of strains were resistant to single antibiotic (AMP), where as the rest of the strains (91.5%) were found to be resistant to four to ten antibiotics. MAR index was in the range of 0.09 to 9.0. Plasmid profile showed two categories (i.e) <10 kb and >23 kb. November of plasmids ranged from one to ten.

Keywords: *Escherichia coli*, EMB agar, Antimicrobial susceptibility testing, Plasmid DNA profiling

Introduction

Escherichia coli is a non-spore forming facultative anaerobe belongs to the family *Enterobacteriaceae*, typically inhabits mammalian intestine, and is therefore widely used as an indicator of fecal pollution of water. Coliform bacteria are described and grouped, based on their common origin or characteristics, as either total or fecal coliform. Total coliform group includes coliforms of other both fecal and non-fecal origin. Fecal coliform bacteria exist in the intestines of warm blooded animals and humans, and are found in bodily waste, animal droppings etc. The prevalence of fecal contamination in water reservoirs has become an important problem in developing as well as developed countries (Raj, 2012).

The increasing use of antibiotics in medical, veterinary and agricultural sectors has almost become synonymous with increased resistance of bacteria to the antibiotics (Dhanorkar and Tambekar, 2004; White, 2006 and Ahmed *et al.*, 2010). Resistant bacteria can be transferred to humans via livestock and through wastewater from slaughter houses, as well as wastewater from hospitals and pharmaceutical plants (Crimmins and Beltran-Sanchez, 2011 and Carlet *et al*., 2012).

E. coli and *Klebsiella* species are commonly found in the environment and the gastrointestinal tracts of wide range of animals (Haryani *et al*., 2007). Commensal *E.coli* can act as a reservoir of resistance genes in the human gut. These resistant genes might be rapidly transferred to other commensal or pathogenic organisms (Salyers *et al*., 2004 and Blake *et al*., 2003). Faecal *E. coli* is regarded as an useful indicator of the spread of acquired antibiotic resistance genes in the community (Lester *et al.*, 1990 and Nys *et al*., 2004).

These resistance genes are commonly present on mobile genetic elements such as plasmids and integrons in clinical isolates of Gram-negative microorganisms (Alekshun and Levy, 2007).

Plasmids are major mechanism for the spread of antibiotic resistant genes in bacterial populations (Fang *et al.*, 2008). Conjugation occurs by F-plasmids that can transfer genes encoded for multiple resistance and mobilize other non-conjugative plasmids to host cells. Multiple resistance genes are harbored on R-plasmids some of which are conjugative (Pitout *et al.*, 2009). In the present study, the antibiotic resistance and plasmid profile of *E. coli* strains isolated from clinical samples were studied.

Materials and Methods

Collection of samples

Clinical samples were collected from hospitals located in Chidambaram, Parangipettai and Cuddalore area using sterile containers and were brought to the laboratory in portable ice cheat maintaining at 4°C. and analyses were made within two hours of collection.

Isolation of *E. coli* from clinical samples

Collected clinical samples were streaked on EMB agar plate for the selective isolation of *E. coli*. The colonies were confirmed by the appearance of greenish metallic sheen on EMB agar plate. The positive colonies on EMB agar plates were streaked on nutrient agar slants and stored at 4^{o}C. The strains were identified according to the biochemical reactions given in Bergey's manual

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Antimicrobial susceptibility testing

Antibiotic susceptibilities of the isolates were determined by both well diffusion and disk diffusion method using Muller Hinton agar and eleven antibiotics namely Ampicillin (AMP) - 25µg, Cefuroxime (CXM) - 30µg, Amoxcillin (AMC) - 30µg, Cefpodoxime (CPD) - 10µg, Cephalexin (CN) - 30µg, Doxycycline (DO) - 30µg, Levofloxacin (LE) - 5µg, Gentamicin (GEN) - 10µg, Ciprofloxacin (CF) - 5µg, Norfloxacin (NX) - 10µg and Ofloxin (OF) - 5µg. The results were interpreted using Clinical and Laboratory Standards Institute criteria (CLSI, 2006).

The multiple antibiotic resistance (MAR) index of each strain was calculated according to the method described by Krumperman (1983) using the formula: a/b, where 'a' represents the number of antibiotics to which a particular isolate was resistant and 'b' the total number of antibiotics tested.

Plasmid isolation

Plasmid DNA profiling of the strains was done by the alkaline lysis method (Sambrook *et al.*, 1989). All bacterial strains were incubated overnight in 5 mL Luria Bertani broth at 37 °C. The bacterial cells were collected by centrifugation at 10000 rpm for 5min in a 1.5 mL micro centrifuge. To the pellet 100 µL of glucose-Tris-EDTA buffer was added and completely re-suspended. To that 200 µL of lysis buffer solution was added and mixed gently for 5 min. The tube was spun at 10000 rpm for 5 min and supernatant was transferred to a sterile eppendorf tube. Equal volume of phenol: chloroform was added and vortexed. They centrifuged at 10 000 rpm for 5 min and the supernatant was transferred to a clean eppendorf tube. Plasmid DNA extracted was precipitated and concentrated using ice cold isopropanol. The resolution of the isolated plasmid DNA was checked on 0.8% agarose gel. 1kb DNA ladder and Lambda DNA Hind III digest marker DNA were used as size standards (Fermentas).

Results

Clinical samples were collected from hospital environments in and around Chidambaram, Parangipettai and Cuddalore. According to morphological and biochemical properties the isolates were identified as *E. coli* species.

A total of 175 *E. coli* strains were used for testing their antimicrobial susceptibility. Regarding susceptibility of *E. coli* strains isolated from clinical samples the resistant and sensitive patterns were tested to eleven antibiotics at a concentration of Ampicillin (AMP) - 25µg, Cefuroxime (CXM) - 30µg, Amoxcillin (AMC) - 30µg, Cefpodoxime (CPD) - 10µg, Cephalexin (CN) - 30µg, Doxycycline (DO) - 30µg, Levofloxacin (LE) - 5µg, Gentamicin (GEN) - 10µg, Ciprofloxacin (CF) - 5µg, Norfloxacin (NX) - 10µg and Ofloxin (OF) - 5µg. Among them 100% resistance was observed for Ampicillin followed by Cephalexin (67.4%), Cefuroxime (59.4%), Cefpodoxime (58.2%), Ofloxin

(54.2%), Amoxcillin (53.7%), Ciprofloxacin (53.1%), Doxycycline (52%), Levofloxacin (49.7%), Norfloxacin (31.4%) and Gentamycin (33.7%) (Table 1 &2).

The MAR index for the isolates was calculated and the range was observed from 0.09-0.9. Among tested isolates for multiple antibiotic resistance (MAR), 15.6% of the strains showed a MAR index of 0.36. Likewise 22% showed 0.46, 15.6% showed 0.55, 22% showed 0.73 and 15.6% showed 0.9 as MAR index.

Table 1: Antibiotic Susceptibility of *E. coli* isolates from clinical samples

Antibiotic	No. of strains Positive out of 175 tested	Resistance %
Ampicillin (AMP)	175	100
Cefuroxime (CXM)	104	59.4
Amoxcillin (AMC)	94	53.7
Cefpodoxime (CPD)	102	58.2
Cephalexin (CN)	118	67.4
Doxycycline (DO)	91	52
Levofloxacin (LE)	87	49.7
Gentamicin (GEN)	59	33.7
Ciprofloxacin (CF)	93	53.1
Norfloxacin (NX)	55	31.4
Ofloxin (OF)	95	54.2

Table 2: Antibiotic resistance pattern, multiple antibiotic resistant index and plasmid profile of *E. coli* isolates from clinical samples

S. No	Antibiotic resistant pattern (ARP)	No. of isolates showed similar ARP	% of resistance	MAR index
1	AMP	15	8.5	0.09
2	AMP-CPD-CN-DO	6	3.4	0.36
3	AMP-CXN-AMC-CPD	3	1.7	0.36
4	AMP-CXN-CPP-NX	5	2.8	0.36
5	AMP-CN-DO-GEN	12	6.4	0.36
6	AMP-CXM-AMC-CPD-CN	5	2.8	0.46
7	AMP-AMC-DO-LE-GEN	3	1.7	0.46
8	AMP-CXM-LE-CF-OF	5	2.8	0.46
9	AMP-AMC-CN-GEN-OF	6	3.4	0.46
10	AMP-CN-DO-GEN-CF	3	1.7	0.46
11	AMP-CXM-CPD-CF-NX	3	1.7	0.46
12	AMP-DO-CF-NX-OF	5	2.8	0.46
13	AMP-CXM-AMC-CPD-CN-DO	8	4.5	0.55
14	AMP-CXM-AMC-DO-GEN-CF	8	4.5	0.55
15	AMP-CXM-DO-LE-CF-NX	5	2.8	0.55
16	AMP-CXM-CN-LE-GEN –OF	5	2.8	0.55
17	AMP-CXM-CPD-CN-DO-GEN	4	2.2	0.55
18	AMP-AMC-CPD-CN-LE-CF-OF	18	10.2	0.64
19	AMP-CXM-AMC-CPN-CN-DO-GEN	5	2.8	0.73
20	AMP-CXM-AMC-CPN-CN-LE-CF-OF	4	2.2	0.73
21	AMP-CXM-CPD-DO-LE-CF-NX-OF	5	2.8	0.73
22	AMP-CXM-CPD-CN-LE-CF-NX-OF	3	1.7	0.73
23	AMP-CN-DO-LE-GEN-CF-NX-OF	5	2.8	0.73
24	AMP-CXM-AMC-CPD-CN-LE-GEN-CP-OF	10	5.7	0.82
25	AMP- CXM-AMC-CPD-CN-DO-LE-CF-NX-OF	24	13.7	0.9

Table 3: Plasmid profile of *E. coli* isolates from clinical samples

Strain	Plasmid size profile (in kb)		
	<10kb	>23kb	Total No. of plasmids
141	--	--	--
67	--	--	--
78	--	--	--
800	--	2	2
157	2(9.5,8 kb)	4	6
189	--	--	--
36	--	2	2
16	--	--	--
20	--	1	2
26	--	1	1
J	--	1	1
P	--	1	1
158	5(8,7.5,7,6, 5 kb)	5	10
75	--	2	2
029	--	1	1
039	--	1	1
133	--	2	2
173	--	--	--
038	--	--	--
044	--	1	1
064	--	2	2
72	--	3	3
73	--	3	3
113	--	--	--
166	--	1	1
168	3(3,4,9 kb)	3	6
150	--	1	1
145	--	2	2
9	--	1	1
42	--	2	2
117	1(8 kb)	2	3
167	3(8,5.5,3 kb)	3	6

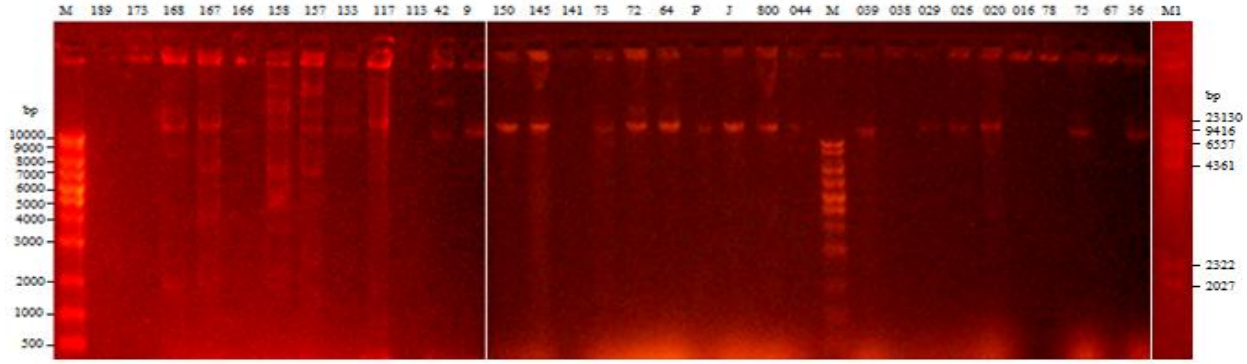

Fig. 1: Plasmid profile of clinical isolates; Lane M: 1kb DNA Ladder (0.5kb to 10kb); Lane M1: Lambda DNA Hind III digest marker, Lanes 189 - 044 and 039 – 36 plasmids from the isolates

Discussion

From clinical samples 175 *E. coli* strains were obtained. Their antimicrobial susceptibility was analyzed. The results of antibiogram showed that 8.5% strains were resistant to one antibiotic where as 91.5% of the strains were resistant to four to ten antibiotics. Regarding resistance to individual antibiotics, 100% resistance was observed towards Ampicillin, followed by Cephalexin (67.4%), Cefuroxime (59.4%), Cefpodoxime (58.2%), Ofloxin (54.2%), Amoxcillin (53.7%), Ciprofloxacin (53.1%), Doxycycline (52%), Levofloxacin (49.7%), Norfloxacin (31.4%) and Gentamycin (33.7%).

Aibinu *et al*., 2004 found 100.0% resistance of *E. coli* isolates to ampicillin. Nsofor and Iroegbu (2013) also observed a high resistance of >75% against Ampicillin. The difference in susceptibility or resistance pattern demonstrated in different geographic locations may be attributable mainly to the usage of those antibiotics for medical, vetenary purpose and also in other fields in that region. In this study, 53.1% of strains were resistant to ciproflaxacin. According to Umolu *et al*., 2006, consistent stepwise increase in *E. coli* resistance to ciprofloxacin was observed from 1995 (0.7%) to 2001 (2.5%). Ciprofloxacin resistance in Portugal was 25.8% and Italy 24.3% while in Germany and Netherlands it was 15.2% and 6.8% respectively (Umolu *et al*., 2006 and Oteo *et al.*, 2007). High resistance of *E. coli* to ofloxacin has been documented by Alex *et al*., 2001. They observed that 24% of 189 *E. coli* isolates were resistant to ofloxacin. Compared to these studies the % resistance observed in the present study was higher to the above antibiotics.

The wide use and abuse of antibiotics in human therapy produce MAR *E. coli* in the feces of humans. These practices have resulted in the coexistence of MAR *E. coli* within these major reservoirs of enteric disease for humans (Feary *et al*., 1972, Isenberg and Berkman. 1971 and Novick, 1981). In the present investigation samples collected from uriene, feces and pus exhibited high MAR index.

In this study, MAR index for the isolates was calculated and the range was observed from 0.09-0.9. Among tested isolates for multiple antibiotic resistance (MAR), 15.6% of the strains showed a MAR index of 0.36. Likewise 22% showed 0.46, 15.6% showed 0.55, 22% showed 0.73 and 15.6% showed 0.9 as MAR index. The isolates with a MAR index of 0.9 were found to be resistant to 10 antibiotics. The higher MAR index of the strains tested showed that the clinical strains are with high MAR index and they may pose threat to the environment if entered in the sewage, storm water (or) directly into river (or) coastal waters.

The choice of an MAR index of 0.200 to differentiate between low- and high-risk contaminations is arbitrary. Indices of between 0.200 and 0.250 are in a range of ambiguity, and samples in this range require careful scrutiny (Krumperman, 1983). In this respect the strains observed in the present study deserves attention. The same author showed that MAR index of *E. coli* isolated from human annal swab was (0.370) and in raw sewage it was 0.630. Maloo *et al*., 2014 observed that 97% of the isolates showed more than 0.2 MAR index. For fecal coliforms and *E.coli*, they observed a MAR index value of 0.05 and 0.04 respectively. Fei *et al*., 2003 observed high multiple antibiotic resistant (MAR) index, ranging from 0.5 to 1.0 for 38 Enteropathogenic *E. coli* (EPEC) strains isolated from diarrhoea patients of Hospital Miri, Sarawak. Similar trend was observed in the present study also.

Study on plasmid profile showed that two types of plasmids were present (i.e) regarding size <10 kb and >23 kb plasmids were found. 75% of the total strains harboured plasmids, where 15.6% of them showed <10kb plasmids. Among the plasmid bearing strains 75% of the strains harboured plasmids of more than 23kb. Strains without plasmids also showed resistance to multiple antibiotics and *Vice versa*. Hence further study is needed to confirm the role of plasmids in acquiring resistance to antibiotics. In another study, clinical isolates of *E. coli* known to harbour plasmids of different molecular size ranging from 2-3 kb to 6.5 kb and maximum 26 kb (Jan *et al*., 2009) which endorsed the results of the present work. Danbara (1987) have also reported the plasmid size between 3.9kb and 50kb in *E.coli* strains isolated from patients suffering from traveler's diarrhoea. The clinical isolates of *E. coli*, along with many others are constantly exposed to the hospital environment where they gain resistance to numerous antibiotics by various mechanisms. This drug resistance increases as a function of time and their exposure to antibiotics as well as due to many other factors.

Nsofor and Iroegbu (2013) observed plasmids of varied sizes. Although, some strains were resistant to only four antibiotics, they had more than one plasmid while others containing one or two plasmids were resistant to a large number of antibiotics. Icgen *et al*., 2002 reported similar findings. In their study, plasmid DNA analysis of the 28 *Salmonella* strains showed that the size of the plasmid DNA was ranged from 3.1 to 32 kb. From their report, most strains associated with non-human sources were found to harbor larger plasmids while most human strains had relatively much smaller plasmids with sizes 10.8, 9, 4.7 and 6.2 kb pairs; yet they were resistant to a larger number of antibiotics. However in the present work, such an observation was not evident as clinical strains harbored both smaller and larger plasmids.

Aja *et al*., 2002 in their study on *Vibrio* strains isolated from cultured shrimps showed some strains resistant to four antibiotics, where others resistant to two antibiotics but all contained single plasmid of 21.2 kb pair. They suggested that resistance to antibiotics could be encoded in some strains in plasmids and in others in the chromosomes. Samething might be true in the present study also. Similarly, Levy (1992) showed that research done with plasmid- and transposon-free *E. coli* was resistant up to seven types of antibiotics, including tetracycline. According to them this higher level of resistance may be caused by initial mutation located in a single site on the *E. coli* chromosome. Antibiotic resistance in pathogens might have evolved from various mechanisms. When it is plasmid mediated, dissemination to other pathogens seemed to be faster. Hence the varying plasmids up to 10 numbers in the present study seemed to be of high risk.

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