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Original Article

Surveillance of Drug Resistance and Plasmid Properties of Clinical Isolates collected from Madurai Region

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Abstract

Curing of infectious diseases depends upon selected use of chemotherapeutic agents or antibiotics. A retrospective study was carried out to determine the current status of drug susceptibility pattern of clinical isolates by Kirby-Bauer method. One hundred and five isolates were collected from clinical laboratories in Madurai, which included *E.coli* (57 /105), *Pseudomonas aeruginosa* (14/105), *Klebsiella pneumoniae* (16/105), *Proteus vulgaris* (02/105) and *Staphylococcus aureus* (16/105). In our present study, Imipenem was found to be more active than any other antibiotics. On the contrary, Penicillin G was found to be inactive against all the clinical isolates. After antibiogram, multidrug resistance isolates were assayed for their plasmid properties. There was no correlation between the plasmid pattern and their antibiogram.
Key words: Clinical isolates, Antibiogram, Plasmid profile.

Introduction

The effective control of any infectious disease depends on prompt diagnosis, isolation of causative agents and understanding their Antibiotic Susceptibility Test (AST) results. The information from epidemiological study provides all information needed to control the infectious diseases (Malhotra *et al.,* 2002).Unresponsiveness to antibiotic therapy is due to resistance of the pathogenic microbes against the drug given. The resistance is contributed by genetical background and environmental condition of the pathogens. Most of the infections like Methicillin-Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant *Staphylococcus aureus* (VRSA), Vancomycin Resistant *Streptococcus fecalis* and Extensively Drug Resistant-Tuberculosis (XDR-TB) are difficult to control and manage at clinical level. The drug resistance is due to many defensive characters which are coded in the chromosome itself or they are coded by extra – chromosomal DNA element called Plasmid, specifically called R – Plasmid. These plasmids are transmissible from one to other bacteria and made them to resist antibiotic treatment.Many workers have reported the presence of *Escherichia coli* harbouring R plasmids and the influence of antibiotics in increasing the number of resistant organisms. (Howe *et al*., 1976; Kiyoshi *et al*., 1981). So it is planed to find out the drug resistance and plasmid distribution of the clinical isolates.

There is a need for an intensified research on the factors responsible for the increasing frequency of bacteria which are multiple drug resistant (Moller *et al*., 1997). Sudha *et al*. (2001) proposed that there is an urgent need of setting up a national quality control laboratory to provide the performance standards, reference Quality Control strains and quality antibiotic discs to ensure reproducible and reliable results. It is also recommended to generate a reliable data to prevent the emerging of antibiotic resistant bacteria by framing the drug policies and protecting indiscriminate use of antibiotics. Our present work would help to explore the trend of antimicrobial resistance.

Materials and Methods

Sample Collection

Clinical isolates were collected from various clinical laboratories of Madurai. The bacteria were isolated from clinical samples such as urine, blood, pus, wound and stool. After plating on a selective medium, isolates were revived and used for antibiogram analysis and plasmid studies. About 105 isolates were obtained, *E.coli* comprises of 57 (54.28%), *Pseudomonas aeruginosa* 14 (13.33 %), *Klebsiella pneumoniae* 16 (15.23 %), P*roteus vulgaris* 2(1.92%) and S*taphylococcus aureus* 16(15.23%). Their identity was confirmed by

staining and biochemical methods (Balows *et al* ., 1991).

In vitro sensitivity assay

The antibiotic susceptibility test for the isolates were done by Kirby-Bauer disc diffusion method as per the recommendations of Clinical Laboratory Standard Institute (CLSI) (Bauer *et al*., 1966). Depending on the size of clear zone formation, the cultures were labeled as sensitive, moderately sensitive or resistant to the array of antibiotics used in the study.

Plasmid analysis

Based on antibiogram analysis, isolates showing more than 70% resistance were selected for plasmid studies. Plasmid isolation was done using the mini-prep method of Sambrook *et al*. (1984).

The isolated plasmids were separated by running on a 0.8% agarose gel along with lambda DNA / EcoR1 –Hind III double digest molecular marker (Genie, India). The electrophoresis was carried out at 50 V / hr. After electrophoresis, the gels were visualized under UV illumination in a Gel-documentation system. The gel images were analyzed using 'Lab Image ID 2006` software.

Results

In our present investigation, the results of *in vitro* sensitivity analysis of *E.coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus vulgaris* to different antibiotics is shown in Table-1 and *Staphylococcus aureus* in Table- 2.

In vitro sensitivity assay on Enterobacteriaceae

Of the total *E.coli* isolates, three of them were resistant to 80% of the antibiotic used in our current experiment. Strain CL3222 and CL3511 were sensitive to Imipenem and Rifampicin, while, strain CL17 is sensitive to Imipenem and Nitrofurantoin. Similarly, strain CL 80 is sensitive to Cephalexin and Streptomycin but resistant to Imipenem. It is obvious from Table 3 that all *E.coli* isolates were resistant to Doxycycline, Tetracycline, Penicillin, Carbenicillin, Ciprofloxacin, Ceftazidime, Meropenem, Co-trimoxazole, Tobramycin, and Nalidixic acid while, sensitive to Imipenem. It was found that Strain CL 3511 alone was resistant to Nitrofurantoin. Strain CL 80 showed resistant against Imipenem. The plasmid profile of *E.coli* is shown in the Fig. 1 and Table 4.

It was found that, 16 strains of *Klebsiella pneumoniae* were isolated from clinical samples and were subjected to *in vitro* antibiotic assay. The plasmid profile of *K. pneumonia* is shown in the Fig -2 and Table- 5.

Two *P. vulgaris* isolates were subjected to antibiotic assay and Imipenem, Ceftazidime, Gentamycin, Amikacin, Netillin and Ciprofloxacin were found to be most effective against *P. vulgaris*. Both the isolates were resistant to Doxycycline, Tetracycline, Penicillin, Carbenicillin, Meropenem, Co-trimoxazole and Streptomycin (Table 1).

In vitro sensitivity assay on Pseudomonas aeruginosa

In the present work, totally 14 *P. aeruginosa* strains were analyzed. The plasmid profile of selected *P. aeruginosa* is shown in the Fig- 3 and Table- 6.

In vitro sensitivity assay on Staphylococcus aureus

In the present study, 16 strains were isolated and subjected to antibiotic sensitivity assay. Compared to Gram negative isolates, *S.aureus* were susceptible to more number of antibiotics. Similar to Gram negative bacteria, they are also 100% susceptible to Imipenem.

The percentage of resistance of the pathogens against the tested antibiotics is shown in the Table-7. It is clear that Imipenem, Amikacin and Vancomycin are potent antimicrobials against pathogenic microbes .

Plasmid DNA analysis

E. coli isolates numbered CL2784, 92, 79,60,89,59 and 3511 were selected for plasmid analysis based on their drug resistant profile. The separated plasmid samples were interpreted by running them on 0.8% agarose gel along with lambda DNA / EcoR1 –Hind III double digest molecular marker (Genie, India). Most of the isolates had a common DNA size of 20.4 Kbp, while Strain CL2784 had a size of 11Kb DNA. Similarly, the plasmid profile of *P. aeruginosa* Strain CL3216, 96, 3225, 2005, 2790, 41 and 2712 and *K. pneumoniae* Strain CL 70, 3506, 3137, 55 and 3229 was selected for their plasmid property analysis. Each *P. vulgaris* isolate showed single DNA molecule after plasmid DNA isolation. Figure 1,2 and 3 show the plasmids isolated from *E.coli, Klebsiella pneumonia* and *P. aeruginosa* respectively.

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Table-1: Levels (%)of resistance, intermediate and sensitivity of Gram negative organisms

Organism		Antibiotics																			
		Do	T	C	P	Cb	Cp	Ca	I	Mr	Co	S	Tb	G	Ak	Nt	Cf	Sc	Na	Nf	R
E.coli (Total - 57)	S	11	2	39	--	1	15	22	55	7	6	23	18	24	41	40	11	12	5	52	28
	%	19.2	3.5	68.4	--	1.7	26.3	38.6	96.5	12.2	10.5	40.4	31.6	42	72	70	19.3	21	8.7	91.2	49
	I	1	6	--	--	1	3	7	--	1	3	16	4	10	13	5	7	3	3	3	8
	%	1.8	10.5	--	--	1.8	5.3	12.4	--	1.8	5.3	28	7	17.6	22.8	9	12.3	5.3	5.3	5.3	14
	R	45	49	18	57	55	39	28	2	49	48	18	35	23	3	12	39	42	49	2	21
	%	79	86	31.6	100	96.5	68.4	49	3.5	86	84.2	31.6	61.4	40.4	5.2	21	68.4	73.7	86	3.5	37
P.aeruginosa (Total - 14)	S	--	--	3	--	--	3	9	13	1	1	4	10	8	10	9	6	6	2	4	2
	%	--	--	21.4	--	--	21.5	64.3	92.9	7.1	7.1	28.6	71.5	57.2	71.5	64.3	42.9	42.9	14.3	28.6	14.3
	I	--	--	3	--	1	--	1	--	--	--	1	--	1	3	1	1	--	--	1	3
	%	--	--	21.4	--	7.1	--	7.1	--	--	--	7.1	--	7.1	21.4	7.1	7.1	--	--	7.1	21.4
	R	14	14	8	14	13	11	4	1	13	13	9	4	5	1	4	7	8	12	9	9
	%	100	100	57.2	100	92.9	78.5	28.6	7.1	92.9	92.9	64.3	28.5	35.7	7.1	28.6	50	57.1	85.7	64.3	64.3
K.pneumoniae (Total - 16)	S	--	--	11	--	1	5	4	16	1	5	5	5	5	10	6	4	5	3	9	3
	%	--	--	68.8	--	6.2	31.2	25	100	6.2	31.2	31.2	31.2	31.2	62.5	37.5	25	31.2	18.8	56.3	18.7
	I	2	2	--	--	--	--	2	--	--	1	5	--	--	4	1	3	--	1	2	4
	%	12.5	12.5	--	--	--	--	12.5	--	--	6.2	31.2	--	--	25	6.2	18.8	--	6.2	12.5	25
	R	14	14	5	16	15	11	10	--	15	10	6	11	11	2	9	9	11	12	5	9
	%	87.5	87.5	31.2	100	93.8	68.8	62.5	--	93.8	62.6	37.6	68.8	68.8	12.5	56.3	56.3	68.8	75	31.2	56.3
P.vulgaris (Total - 2)	S	--	--	--	--	1	1	2	2	--	--	--	2	2	2	2	2	2	2	--	1
	%	--	--	--	--	50	50	100	100	--	--	--	100	100	100	100	100	100	100	--	50
	I	--	--	--	--	--	1	--	--	--	--	--	--	--	--	--	--	--	--	1	1
	%	--	--	--	--	--	50	--	--	--	--	--	--	--	--	--	--	--	--	50	50
	R	2	2	2	2	1	--	--	--	2	2	2	--	--	--	--	--	--	--	1	--
	%	100	100	100	100	50	--	--	--	100	100	100	--	--	--	--	--	--	--	50	--

(Number of isolates S-Sensitive, I-Intermediate, R-Resistant)
% - row indicate isolate`s percentage of response against antibiotics

[Doxycycline(DO)- 30mcg, Tetracycline(T) - 30mcg, Chloramphenicol(C) - 30mcg, Penicillin G(P)-10 units, Cloxacillin(Cx) - 10mcg, Carbenicillin(Cb) - 100mcg, Cephalexin(Cp) -30mcg, Ceftazidime(Ca)- 30mcg, Imipenem(I)- 10mcg, Meropenem(Mr)- 10mcg, Co-trimaxazole (Co) - 25mcg, Streptomycin(S) -10mcg, Tobramycin(Tb) -10mcg, Gentamicin(G) -10mcg, Amikacin(A) -30mcg, Netillin(Nt) -30mcg, Ciprofloxacin(Cf)- 5mcg, Sparfloxacin(Sc) -5mcg, Nalidixic acid(Na) -30mcg, Nitrofurantoin(Nf) - 300mcg, Rifampicin(R) -5mcg,]

Table-2: Levels (%) of resistance, intermediate and sensitivity of *S. aureus*

	Do	C	P	Cx	Ox	Cp	Ca	I	Mr	Co	E	S	G	AK	Nt	Cf	Sc	Va	R
S	43.7	93.7	-	-	81.3	19	31	100	62.5	18.7	68.7	81.2	31.2	56	93.7	25	37.5	100	81.3
I	12.5	-	-	18.7	-	56	43	-	6.25	12.5	6.25	6.25	6.25	25	-	31.2	18.7	-	-
R	43.7	6.25	100	81.2	18.7	25	25	-	31.2	68.7	25	12.5	62.5	19	6.25	43.7	43.7	-	18.7

(S-Sensitive, I-Intermediate, R-Resistant)

[Doxycycline(DO), Chloramphenicol(C), Penicillin G(P), Cloxacillin(Cx), Oxacillin(Ox)-1mcg, Cephalexin(Cp), Ceftazidime(Ca), Imipenem(I), Meropenem(Mr), Co-trimaxazole (Co), Erythromycin(E)- 15mcg, Streptomycin(S), Gentamicin(G), Amikacin(A), Netillin(Nt), Ciprofloxacin(Cf), Sparfloxacin(Sc),Rifampicin(R),Vancomycin(V)-30mcg] .

Table-3: Sensitivity pattern of selected *E.coli* isolates to different antibiotics.

Isolates (CL)	Name of drugs																			
	Do	T	C	P	Cb	Cp	Ca	I	Mr	Co	St	Tb	G	Ak	Nt	Ct	Sc	Na	Nf	R
2784	R	R	S	R	R	R	R	S	R	R	R	R	S	S	S	S	S	R	S	R
92	R	R	S	R	R	R	R	S	R	R	I	R	R	I	R	R	R	R	S	S
79	R	R	R	R	R	R	R	S	R	R	I	R	R	S	I	R	R	R	S	S
60	R	R	R	R	R	R	R	S	I	R	S	R	R	I	R	R	R	R	S	S
89	R	R	S	R	R	R	R	S	R	R	S	R	R	I	R	R	R	R	S	S
59	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	I	R	I	S	R
3511	R	R	R	R	R	R	R	S	R	R	R	R	R	I	I	R	R	R	R	S

(S-Sensitive, I-Intermediate, R-Resistant); [Doxycycline(DO), Tetracycline(T), Chloramphenicol(C), Penicillin G(P), Cloxacillin(Cx), Carbenicillin(Cb), Cephalexin(Cp), Ceftazidime(Ca), Imipenem(I), Meropenem(Mr), Co-trimaxazole (Co), Streptomycin(S), Tobramycin(Tb), Gentamicin(G), Amikacin(A), Netillin(Nt), Ciprofloxacin(Cf), Sparfloxacin(Sc), Nalidixic acid(Na), Nitrofurantoin(Nf), Rifampicin(R)]

Table- 4: Shows plasmid profile of selected *E.coli* strains

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
Strains (CL)→	Marker	2784	92	79	60	89	59	3511
Plasmid Size (pb)	21226 5148 4268 3530 2027 1904 1584 1375 947 831	11501 7709 3633 3203 1904 1521	20421 11501 5765 4142 2321 1432 906	20421 1432 906	20421 11501 3439 3275 2094 1904 1432 906	20421 18023 12541 9561 5765 3637 3203 3059 2027 1816	21226 11164 3439 3275 2094 1904 1521 1288 986	22171 11501 3530 2631

Table- 5: Shows plasmid isolated from selected *Klebsiella pneumonia*

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
Strain Number (CL)→	Marker	70	3506	3137	55	3229
Size of the plasmid (bp)	21226 5148 4268 3530 2027 1904 1584 1375 947 831	19594 2340 1375	21226 1375	21226 1704 1128	10274 5085 2715 1439	21226 10274 5085 2621 1439

Table-6: Shows size of plasmids isolated from selected *P. aeruginosa*

ll	Lane 1	Lane2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
Strain number (CL) →	Marker	3216	96	3225	2005	2790	41	2712
Size of the plasmid (bp)	21226 5148 4268 3530 2027 1904 1584 1375 947 831	21226 4268 2536 1584 1351 675	21226 10797 3806 1351 1004 697	21226 3806 2268 1458 697	21226 1528	21226 3261 1528	21226 4037 2375 1351	21226 9058 3261 1351

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Discussion

Clinicians use antibiogram data to understand drug resistance trends, identifying outbreaks, developing quality improvement initiatives and forming infection control policies and protocols. Our present study investigates the multi-centre study undertaken to evaluate susceptibility patterns of bacterial strains isolated from different clinical samples in Madurai region, Tamil Nadu. It provides valuable laboratory data concerning both community and hospital pathogens and enables the situation in Madurai to be compared with that in other places.

Many authors have reported the drug susceptibility patterns of clinical isolates (Howe *et al* ., 1976 ; Kiyoshi *et al*., 1981; Idia *et al*., 2006). There was a high incidence of *E. coli* than any other pathogens in clinical samples. Imipenem, which belongs to the group called Carbapenem, is most active drug against all type of pathogens. Doxycycline, Tetracycline, Penicillin, Co-trimoxazole, Meropenem, Nalidixic acid and Carbenicillin is less active against these Gram negative isolates.

Among the antibiotics tested Imipenem (96.5%) and Nitrofurantoin (91.2 %) were few to be highly inhibitory to *E.coli*. Similar report was given by Idia *et al.,* (2006) stating that in addition to Imipenem, Co-trimaxazole had maximum inhibition over *E.coli*. The reason for such high resistance to commonly used antibiotics could be due to widespread and indiscriminate use of antibiotics. In this present study isolates were highly sensitive to Nitrofurantoin. Similarly, extreme sensitivity of *E. coli* isolates to Nitrofurantoin has earlier been reported by Bonten *et al*. ,(1990).

In recent years, use of Fluoroquinolones has increased in many countries and emergence of resistance of bacterial isolates to Fluoroquinolones have been observed in different geographical regions. There was a constant increase in *E. coli* resistance to Ciprofloxacin observed from 1995 (0.7%) to 2001 (2.5%) in different countries (Bolon *et al*., 2004). Ciprofloxacin resistance in Portugal was 25.8% and Italy 24.3% while in Germany and Netherlands it was 15.2% and 6.8% respectively (Oteo *et al*., 2005). But in our present study, *E. coli* isolates reported 50% to 86 % resistance to Fluoroquinolone drugs.

Table-7: Shows the percentage of resistance of pathogen against antibiotics

Group		Antibiotics	% resistance
Tetracyclines		Doxycycline	78.09
		Tetracycline	88.76
Amphenicols		Chloramphenicol	32.38
Beta lactams	a) Penicillins	Penicillin G	100
		Cloxacillin	81.2
		Oxacillin	18.7
		Carbenicillin	94.38
	b) Cephalosporins	Cephalexin	61.9
		Ceftazidime	47.19
	c) Carbapenems	Imipenem	0.28
		Meropenem	80
Sulphonamides and trimethoprim		Co-trimaxazole (Trimethoprim/ Sulphomethaxaole)	80
Aminoglycosides		Streptomycin	35.23
		Tobramycin	56.17
		Gentamicin	46.66
		Amikacin	8.5
		Netillin	24.76
Quinolones		Ciprofloxacin	59.04
		Sparfloxacin	64.76
		Nalidixic acid	82.02
Glycopeptides		Vancomycin	0
Macrolides		Erythromycin	25
Others		Rifampicin	40
		Nitrofurantoin	19.10

All the *S. aureus* isolates were sensitive to Imipenem and Vancomycin and 93.75% isolates were to Chloramphenicol and Netillin. Of the total 16 *S. aureus* isolates, 18.25% isolates were resistant to Oxacillin, Streptomycin and Rifampicin. They were also sensitive to Erythromycin (68.75%). Our findings were similar to that of Dar *et al.* (2006), inwhich zero percent resistance to Vancomycin and high resistance to Penicillin were observed.

MRSA is a serious public health problem in world wide. Methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for several difficult-to-treat infections in humans. Three of the *S. aureus* isolates showed resistance to Oxacillin, and there is a reference stating that Oxacillin is preferred as the representative of the β– lactamase resistant Penicillins class of antibiotics which include Methicillin (McDougal and Thornsberry, 1984).

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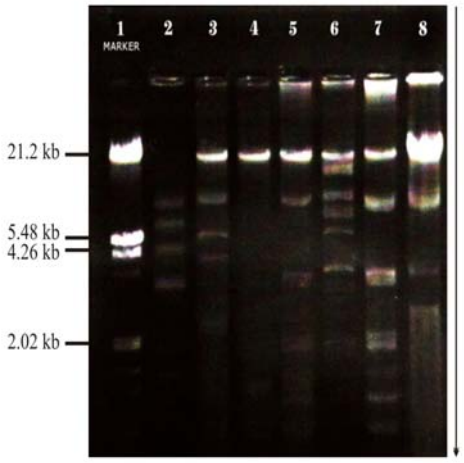

Fig-1: Shows the plasmid profile of selected *E.coli*

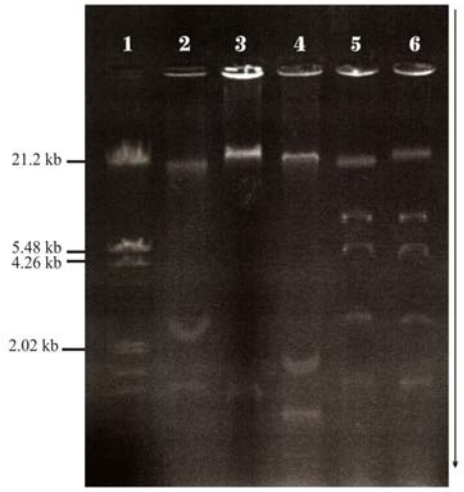

Fig-2: Shows the plasmid profile of selected *Klebsiella pneumonia* strains

From the results, it is clear that, Imipenem is the only antibiotic that is effective against all organisms (both Gram positive and Gram negative) tested. Though Imipenem and Meropenem belong to the same Carbapenem group of antibiotics, there is an extreme resistance to Meropenem by all the group of organisms except *S.aureus*. The resistance to Meropenem by most of the clinical isolates was also documented by Prakash (2006). The role of Imipenem in the treatment of MRSA infections has been studied. Though Imipenem is very active against Methicillin-Sensitive *Staphylococcus aureus* (MSSA), is found to induce resistance in MRSA. There is also a wide disparity in Imipenem MICs between MRSAs and MSSAs. Despite this, Imipenem has been shown to be an effective agent in the treatment of both MRSA and MSSA infection (Lin, 1989).

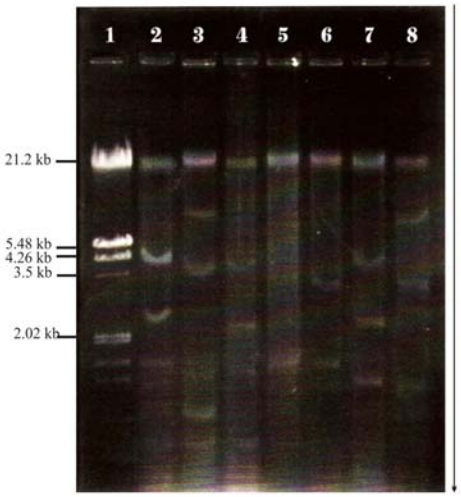

Fig-3: Shows the plasmid profile of selected *P. aeruginosa* strains

Isolates that showed Multidrug resistance were found to harbour plasmid with size ranging from 906 bp to 20,421 bp in the case of *E.coli*, 675 bp to 21,226bp in *P.aeruginosa*; 1,128 to 21,226 bp in *K. pneumoniae*. The results are similar to that of Idia *et al*., (2006). Smith *et al*., (2003) have reported that 47 of the *E. coli* isolated from animals in Lagos harbour detectable plasmids ranging from 0.564kb to 23kb. This indicates that animals could be a source of dissemination of this plasmid resistant *E.coli* in the environment. Danbara *et al.,* (1987) also reported plasmids of sizes between 3.9kb and 50kb in *E. coli* strains isolated from Traveller's diarrhoea. Similarly, Todorova *et al*. (1990) reported that 92% of *E. coli* serotype O164 strain possessed two small plasmids of molecular size 0.906kb and 7.248kb.

In the present study, multi drug resistant *E.coli* isolates were found to harbor 20.4 Kbp plasmid. *E.coli* strain CL89 harbored maximum of 10 plasmids and Strain CL79 showed minimum of 3 plasmids. Both isolates showed more similarities in their antibiogram but their plasmid profile is entirely different from each other. Similar results were described by Karbsizaed *et al.,* (2003). They suggested that there was no constant correlation between plasmid profiles and antibiotic resistance pattern. This may not be true, since the

antimicrobial resistance pattern can also be encoded by unrelated plasmids, transposons, phages and chromosomal genes. Antibiotic resistance patterns and plasmid profiles are sometimes inadequate to clarify the relationships among different clinical isolates from a single hospital and can lead to erroneous epidemiological conclusion.

Conclusion

In the modern era antimicrobial resistance is constantly increasing, there is a need for a regular antimicrobial sensitivity surveillance to compact the health problem in the society. Thus, a national wide survey of clinical isolates and their antibiogram results are essential and should be made available to all. This will help the physicians to provide safe and effective therapeutic strategies to reduce malti-drug resistant infections.

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