CHAPTER V
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PLASMID PROFILE OF SELECTED BETA LACTAMASE PRODUCING STRAINS

The widespread clinical use of antibiotics has mobilized latent mechanisms to neutralize their impact. This process is facilitated by the independent genetic elements that can harbour resistant genes, i.e., bacterial chromosome and plasmids. Interspecies and intraspecies transfer of genetic elements facilitates resistance. Antibiotic resistant plasmids (R-Plasmids) are well known as carriers for spread of antibiotic resistant genes. ESBL and AmpC beta-lactamase genes are typically located on plasmids. So we tried to find out the plasmid profile of some of the selected beta-lactam resistant strains. Small plasmids of sizes 2 kb to 6 kb and big plasmids in the range of 53 kb were isolated from the selected strains.

Plasmids are mobile, extra-chromosomal genetic elements. They can be spontaneously lost or readily acquired by a host strain. The strong selective pressure of antibiotics on the hospital organisms cause selection of antibiotic-resistant bacteria existing in the wild population or force them to acquire the resistance genes from the environment or from other bacteria. Plasmids help spread of resistance amongst different strains as well as among different species, and may cause such plasmids to spread rapidly amongst strains and even among different species and to persist for long periods within a hospital. Genes that mediate resistance can be found on transferable plasmids or on transposons. Some transposons or plasmids have genetic elements termed integron that enable them to capture exogenous genes. A number of genes may insert into a given integron, resulting in resistance to multiple antimicrobial drugs (Gold et. al. 1996). The most frequently encountered hospital pathogens have the ability to acquire and disseminate resistance genes (Davis 1994). The development of bacterial strain that are resistant to antibiotics has emerged as a global problem (Bennett and Brachman 1998).

Resistance plasmids are transferred between bacterial strains generally by the process of conjugation. The ability of conjugation to mediate resistance plasmid
transfer among different bacterial species is of clinical importance. The phenomenon facilitates the spread of resistance genes among different genera and species of bacteria. Another clinically important property of resistance plasmid is their stable inheritance in the absence of antibiotic-mediated selection.

Beta-lactam drugs include penicillins, cephalosporins, monobactams, carbapenem and others. Plasmid-mediated resistance to beta-lactam agents could be due to beta-lactamases, that is, enzymes that hydrolyze the beta-lactam ring, detoxifying the drug (Tenover et al. 1998).

Most of the antimicrobial drugs currently in use are derived from soil organisms, mainly fungi and actinomycetes. The resistance genes probably evolved in the antibiotic producers as part of the biosynthetic gene cluster to protect the producing organism from the detrimental action of its own antibiotic. Subsequent gene transfer events might have spread the resistance determinants to other bacteria (Davies 1994). Plasmid medicated drug resistance is quite common in the nosocomial isolates of *K. pneumoniae*. The plasmid which varied in size from 40-65.8 megadaltons could transfer 4 to 9 drugs resistance including third generation cephalosporins (Tenover et al. 2003). In India a study from Pune isolated a large plasmid of 98.7 kb from *K. pneumonia* (Mishra et al. 2001). The incidence of antibiotic resistance due to acquisition of R-plasmids in intestinal *E. coli* has been reported from Patiala (Kanta et al. 1991). A principal mechanism for the rapid spread of antibiotic resistance genes through bacterial population is that such genes get collected on plasmids that are independently replicated within and passed between bacterial cells and species.

5.2. Materials

For plasmid isolation by the method of Kado and Liu (modified):

The following chemicals were used: Tryptic Soya Broth (Difco laboratories), Tris base (Spectrochem Pvt ltd), Glacial Acetic acid (Qualigen), EDTA (Merck), SDS (Qualigen), NaOH (Qualigen), Phenol (Genei), Chloroform (Qualigen), Ethydiu bromide (Sigma), Agarose (BDH Laboratory) and Distilled water.
5.3. Method

Plasmid Isolation:
The modified alkaline lysis method of Kado and Liu was as followed. Slants prepared with Mueller Hinton Agar were taken having the required bacterial growth. A single colony grown on Luria Agar plate was taken and inoculated into 10 ml Luria Broth and it was then incubated overnight at 37°C in a rotary shaker. 1.5 ml of the culture was taken and transferred to 1.5 ml microfuge tube. It was then centrifuged at 12000 rpm at 4°C for 3 minutes. The supernatant liquid was discarded and the tube was inverted on the tissue paper. Once the pellet was dry, 100 μl of lysing solution (0.15g Tris and 0.75 SDS dissolved in 23 ml distilled water in which 1.5 ml of 2N NaOH was added) was added and the pellet was suspended gently using microtips. This suspension should be homogenous. The tube was incubated at 60°C for 5 min in water bath. Equal volume of phenol chloroform mixture (1:1) was added immediately and mixed well by inverting the tubes. It was then centrifuged at 12000 rpm at 4°C for 15 min. The aqueous phase was then applied to horizontal Agarose gel (0.8wt/vol) electrophoresis. Electrophoretic run was carried out at 12 Vcm⁻¹ for 4 hours in TAE buffer [48.4 g Tris(0.04 M), 11.42 ml Glacial acetic acid, 20 ml EDTA(0.5M) pH 8.2], gel was stained with Ethidium bromide (1 μg/ml) (Kado et. al. 1981).

5.4. Results and Discussion

The first plasmid-mediated beta-lactamase in Gram-negatives, TEM – 1, was described in early 1960s (Datta 1965). The beta-lactamase producing genes, especially ESBL genes are frequently located on plasmids and are readily transferable among species (Bush et. al. 1995). Of the different plasmid-mediated beta-lactamases recorded in Gram-negative bacilli the commonest of these enzymes in Enterobacteria is TEM – 1, which is responsible for most of the ampicilin resistance seen in about 50% of E-coli isolates (Sanders et. al. 1992). It has been reported from Nigeria that ESBL producing K.pneumoniae are associated with large plasmids (≥ 58 kb) which is carried by bla(CTX-M) gene along with tetracycline resistance gene and various aminoglycoside genes (Soge et. al. 2006). Some of the representative ESBL producing isolates were examined by us for the presence of plasmid DNA.
We examined four *E. coli* (*E. coli* CMC-33, *E. coli* CMC-59, *E. coli* CMC-35, *E. coli* CMC-20) and three *K. pneumoniae* (*K. pneumoniae* CMC-19, *K. pneumoniae* CMC-99, *K. pneumoniae* CMC-41) ESBL producing isolates for analysis of plasmid. All of these strains showed the presence of a large plasmid >53.4 kb and small plasmids of sizes 2.1 kb to 6 kb (Fig 14). *E. coli* V517 (Macrina FL *et al.* 1978) having plasmids of sizes 53.4kb, 5.5kb, 5.1kb, 3kb, 2.7kb, and 2.1kb was included as reference strain (Lane 8). It is reported that ESBLs production is coded by genes that are located on large conjugative plasmids of size 80 kb to 160 kb (Steward CD *et al.* 2001). We found the maximum size of plasmid in our ESBL producing isolates (Fig 14) to be just greater than 53.4 kb. We, as yet could not confirm that these plasmids were harbouring beta-lactamase gene and that they were responsible for beta-lactamase production.

Plasmid-mediated AmpC beta-lactamase were detected in the late 1980s, providing mobile AmpC beta-lactamases to species such as *Klebsiella* *spp.*, *Salmonella* *spp.*, *C. freundii*, *E. aerogenes*, *P. mirabilis* and *E. coli* (Livermore 1995, Philippon 1994). Usually encoded on large plasmids which also contains genes encoding resistance to other drug classes, these enzymes can be associated with resistance to all antibiotics except carbapenem, fourth generation cephalosporins, and fluoroquinolones (Thomson *et al.* 2000). The genes encoding AmpC enzymes are thought to have been relocated from chromosomes onto plasmids and then transmitted from the original host species to other species. In 1989, Bauernfeind *et al.* described a *K. pneumoniae* isolate from South Korea that could transfer resistance to cefoxitin and cefotetan as well as penicillin, oxyimino-cephalosporins, and monobactams to *E.coli* (Bauernfeind *et al.* 1989). The enzyme, termed CMY-1 for its cephaprycinase activity, had an isoelectric pI of 8 and was more sensitive for inhibition by sulbactam than by clavulanic acid and tazobactam, suggesting that it might be AmpC beta-lactamase enzyme. However, the first proof that a AmpC beta-lactamase had been captured on a plasmid was provided be Papanicolaou *et al.*., (Philippon *et al.* 2002) who described transmissible resistance to α-methoxy- and oxyimino-beta-lactams mediated by an enzyme (MIR-1). Hence the first reported plasmidic AmpC beta-lactamases were MIR-1 in Providence, RI, USA (Papanicolaou *et al.* 1990) and CMY-1 in Seoul, South Korea, both produced by isolates of *K. pneumoniae* (Bauernfeind *et al.* 1989).

We examined three *E.coli* (*E.coli* CMC-28, *E.coli* RGK-2, *E.coli* CMC-100), two *K. pneumoniae* (*K. pneumoniae* CMC-242, *K. pneumoniae* CMC-10) and
one *P. aeruginosa* CMC-3, AmpC producing isolates and one inducible AmpC producing strain *K. pneumoniae* CMC-40 for the presence of plasmid DNA. All three *E. coli* isolates (Lane 2, 3, 4), *K. pneumoniae* CMC-10 (Lane 6) and the inducible AmpC producer *K. pneumoniae* CMC-40 (Lane 8) showed a large plasmid ≥ 53.4 kb (Fig 15). Small plasmids of size 2.7 kb to 6 kb were observed in *E. coli* RGK-2 (Lane 2), *E. coli* CMC-100 (Lane 4) and in *K. pneumoniae* CMC-10 (Lane 6). In *K. pneumoniae* CMC-242 (Lane 5) and in *P. aeruginosa* CMC-3 (Lane 7) plasmids were not visible in our hands with the method followed by us. *E. coli* V517 (Macrina *et al* 1978) was used as reference strain.

Although there is a report of plasmid-mediated inducible DHA-2 beta-lactamase from Taiwan which had a 70 kb plasmid (Yan *et al*. 2002) but the inducible AmpC beta-lactamase producing strain in our study showed a plasmid having a size slightly greater than 53.4 kb. Reports from Japan (Yamoda *et al*. 2003) showed large plasids of size 98 kb in Metallo-beta-lactamase producing *Pseudomonas putida*. We could not find any plasmid in our MBL strains either because of absence of plasmids or because the method used is unable to detect large plasmids.

It is likely that all “plasmid-mediated” beta lactamases have chromosomal origins, although the source organism for many types remains unknown. The distribution of the plasmid mediated enzyme reflects the transmissibility of the elements to which their genes have spread. In almost all beta-lactamase producing strains tested by us we have observed a large plasmids ≥ 53.4 kb. In all probability these plasmids have got resistant marker genes of specific type which may be explored in future.
Fig 14: Agarose gel electrophoresis pattern of seven representative ESBL strains and one reference strain.

Lane 1: *E. coli* CMC-33
Lane 2: *E. coli* CMC-59
Lane 3: *E. coli* CMC-35
Lane 4: *E. coli* CMC-20
Lane 5: *K. pneumoniae* CMC-19
Lane 6: *K. pneumoniae* CMC-99
Lane 7: *K. pneumoniae* CMC-41
Lane 8: *E. coli* V517 (Plasmid containing reference strain, having plasmids of sizes 53.4kb, 5.5kb, 5.1kb, 3kb, 2.7kb, and 2.1kb).

Chr: indicates chromosomal band in plasmid DNA preparations
Fig 15: Agarose gel electrophoresis pattern of six representative AmpC beta-lactamase strains and one Inducible ampC producing strain and one reference strains.

Lane 1: *Escherichia coli* V517
Lane 2: *E. coli* CMC-28
Lane 3: *E. coli* RGK-2
Lane 4: *E. coli* CMC-100
Lane 5: *K.pneumoniae* CMC-242
Lane 6: *K.pneumoniae* CMC-10
Lane 7: *P.aeruginosa* CMC-3
Lane 8: *K.pneumoniae* CMC-40 (Inducible AmpC producing strain)
Lane 9: *E. coli* V517 (Plasmid containing reference strain, having plasmids of sizes 53.4kb, 5.5kb, 5.1kb, 3kb, 2.7kb, and 2.1kb).

Chr: indicates chromosomal band in plasmid DNA preparations.

*E. coli* NRS-20