


Intraspecies transfer of a chloramphenicol-resistance plasmid of staphylococcal origin

Mausumi Bhakta*, Suranjana Arora & Manjusri Bal

Section of Microbiology, Department of Physiology, University College of Science & Technology, University of Calcutta, Kolkata, India

Received November 6, 2002

Background & objectives: The emergence of antibiotic-resistant bacteria is a phenomenon of concern to the clinician as well as to the pharmaceutical industry, because it is the major cause of failure in the treatment of infectious diseases. The genetic exchange of plasmids containing antibiotic resistant determinants (R-plasmids) between organisms of the same or different species is believed to play a crucial role in the evolution of antibiotic resistant bacteria. *Staphylococcus aureus* is well known for its multi-drug resistance (MDR). This work was undertaken to study the intraspecies transfer of a chloramphenicol (C) resistance staphylococcal R-plasmid among different clinical isolates of *S. aureus*.

Methods: From a MDR *S. aureus* MC524 strain, a small plasmid pMC524/MBM was isolated. Lysostaphin lysis and sucrose mediated detergent lysis were used for plasmid preparation. Agarose gel electrophoresis, transformation experiments, Southern blotting and hybridization were done. Restriction endonuclease (RE) digestions were performed.

Results: pMC524/MBM, which codes for C resistance could be transferred into some C sensitive clinical strains of *S. aureus*. The size and the RE digestion patterns of the plasmids isolated from the *S. aureus* transformants were identical to those of pMC524/MBM.

Interpretation & conclusion: These results suggest that pMC524/MBM, without any modification is capable of transferring, maintaining, replicating and expressing itself in different clinical strains of *S. aureus* and hence may be responsible for the spread of C resistance.

Key words: Antibiotic resistance plasmid - chloramphenicol resistance plasmid - intraspecies plasmid transfer plasmid transformation - staphylococcal plasmid

The development and spread of bacterial strains that are resistant to antibacterial drugs has emerged as a global problem [1]. The transmission of antibiotic resistance genes from streptomycetes and other soil bacteria to clinically important species may have occurred through the exchange of antibiotic resistance plasmids (R-plasmids) or transposons during the last 50 yr of widespread antibiotic use and therapy [2]. The appearance of antibiotic resistant bacteria over the past decades has been regarded as an inevitable genetic response to the strong selective pressure imposed by antimicrobial chemotherapy, which plays a crucial role in the evolution of antibiotic resistant bacteria. These bacteria then pass
the antibiotic resistance plasmid among other bacterial cells and species.

With a view to understand the spread of antibiotic resistance in bacteria, we studied the antibiotic resistance plasmid of Staphylococcus aureus, a Gram positive pathogen responsible for 9 to 10 per cent of all nosocomial infections world-wide. More than 51 per cent of all S. aureus isolates from clinical sources from Kolkata were found to be multiple antibiotic resistant. Plasmid mediated antibiotic resistance in S. aureus has been reported from many laboratories.

We isolated a multiple antibiotic resistant clinical S. aureus strain MC524, resistant to ampicillin (A), chloramphenicol (C), kanamycin (K) and streptomycin (S), which possessed a single plasmid and we designated this as pMC524/MBM. In this paper, we report some of the characteristics of this plasmid, and its capability to transfer itself to different clinical strains of S. aureus.

Material & Methods

Bacterial strains: S. aureus MC524 (phage type 47,95 RTD) strain was isolated from a sample of pus from an indoor patient admitted to the Calcutta Medical College & Hospital (CMCH), Kolkata in 1996. Other chloramphenicol sensitive plasmidless clinical strains of S. aureus were collected from CMCH, Nil Ratan Sarkar Medical College, Bangur Hospital, RG Kar Medical College and School of Tropical Medicine, Kolkata. The investigations using these strains were carried out in the Section of Microbiology, Department of Physiology, University of Calcutta, during 1996-2002. S. aureus RN4220, a plasmidless, all antibiotic sensitive and restriction deficient strain and a derivative of S. aureus NCTC 8325 suitable as a transformation host was a gift from Dr Richard P. Novick, Skirball Institute, New York, USA.

Media & chemicals: The S. aureus strains were maintained on TSBYEG medium [3% Trypticase soya broth (TSB, Hi-media, India), 0.3% yeast extract (Oxoid Ltd, UK) and 0.5% glycan (Sigma, USA)]. For S. aureus protoplast transformation, Penassay (PA) broth [0.368 g K$_2$HPO$_4$ (Merck, India), 0.132 g KH$_2$PO$_4$ (Merck, India), 0.15 g yeast extract, 0.5 g bacitriepentone (Difco Lab, USA), 0.35 g sodium chloride (Qualigens Fine Chemicals, India), 0.1 g dextrose (Sarabhai M Chemicals, India), 0.15 g beef extract (SD Fine Chemicals Ltd, Bolsar), water 100 ml, pH 7] was used. S. aureus transformants were selected on DM3 regeneration medium containing 135 g/l sodium succinate (Sisco Research Lab Pvt Ltd, India), 5 per cent w/v casamino acids (100 ml/l, Difco, USA), 20 per cent w/v glucose (25 ml/l, BDH Lab, India), 10 per cent w/v BSA (10 ml/l, Sigma, USA), agar (8 g/l, Merck, India), and the volume made to 1000 ml with water. Sodium succinate was used as an osmotic stabilizer. Antibiotics were added as required.

Antibiotic susceptibility pattern: Determination of susceptibility of the donor and the transformant strains to 8 different antibiotics, i.e., A, C, S, K, erythromycin (E), gentamycin (G), methicillin (M) and tetracyclin (T) was determined by the disc agar diffusion technique (DAD). Minimum inhibitory concentration (MIC) of different antibiotics was determined by the serial 2-fold broth dilution method. Both DAD and MIC were determined in Muller Hinton Broth (MHB, Hi-media, India). A, C, S, K, E, G were products of Sigma, USA, M and T were gifts from Dr M.K. Mazumdar, ex-Director, Central Drug Laboratory, Kolkata.

Plasmid isolation: Plasmid DNA from S. aureus was isolated both by lysostaphin lysis and sucrose mediated detergent lysis methods.

Restriction endonuclease (RE) digestion: The plasmids were digested with BamH I, EcoR I, Hind III and Pst I (Bangalore Genei), according to the instructions of the manufacturers.

Protoplast transformation: Transformation of S. aureus RN4220 (a restriction modification minus plasmidless strain suitable for transformation) was performed by the protoplast transformation method with slight modification. The host S. aureus RN4220 was grown in 2xPA broth to mid log phase (5x10$^8$ cells/ml) and protoplasts were prepared by
incubating the mid log phase cells with lysostaphin (Sigma, USA) at 37°C for 20 min, stabilized with glucose (Qualigens Fine Chemicals, India) and Mg²⁺ or transformation, 30-40 ng of plasmid DNA in a volume of 0.25 ml was mixed with an equal volume of SMM [sucrose 324 g/l, sodium maleate 4.6 g/l and MgCl₂ 8.1 g/l (Qualigens Fine Chemicals, India)]. 0.5 ml of protoplast suspension was added and the mixture was shocked with polyethylene glycol (PEG, Sigma, USA). Protoplasts were plated into DM3 regeneration medium containing the appropriate antibiotic and incubated at 37°C. Colonies began to appear after 48 h. In this study A, S or K containing DM3 medium was used.

**Southern blotting and hybridization:** Plasmid DNA was electrophoresed on 0.8 per cent w/v agarose gel (Sigma, USA) in Tris-Borate-EDTA (TBE) buffer (Sigma, USA), and then transferred to Hybond membrane (Amersham, USA). pMC524/MBM was radioactively labelled with [α³²P] - dCTP (Bhabha Atomic Research Centre) by the random priming method using Gibco BRL Random Primers Labelling System. Prehybridization was carried out at 65°C. Subsequent washings and signal development were done following the method of Lambrook et al.¹⁹

**Results**

*S. aureus* MC524 was resistant to A, C, K and S by the disc agar diffusion technique. Its MIC value for chloramphenicol in MHB, was found to be 25 μg/ml.

Plasmid analysis of *S. aureus* MC524 revealed that it harboured a small plasmid, designated as pMC524/MBM. This plasmid has a single cutting site for Hind III, but no cutting sites for EcoRI, BamHI or PstI. Fig. 1 shows the RE digestion pattern of pMC524/MBM. Lane 2 in Fig. 1 shows linearized form of pMC524/MBM due to single cut by Hind III.

Transformed *S. aureus* RN4220 clones with plasmid pMC524/MBM were selected on DM3 plates containing either A or K or S. It was thus concluded that pMC524/MBM codes for chloramphenicol resistance.

Ten, chloramphenicol sensitive, plasmidless clinical isolates of *S. aureus* different from each other as established by phage typing were procured from different Kolkata hospitals stocks, and attempts were made to transform these strains with pMC524/MBM following the protoplast transformation method. In seven of the 10 strains, chloramphenicol resistant transformants appeared. Plasmid preparations from these transformants revealed the presence of a small plasmid. Both the electrophoretic mobility (Fig. 2) and RE digestion pattern of these plasmids were identical to those of pMC524/MBM. As pMC524/MBM is a small plasmid the electrophoretic mobility of pMC524/MBM along with the plasmids obtained from two of the transformants, were compared to that of pBR 322 (Fig. 3). Antibiotic susceptibility testing by the disc agar diffusion technique showed that *S. aureus* MC524 (donor) was resistant to A, K, C and S, while the *S. aureus* RN4220/524 transformants and all the transformed clinical isolates were resistant to chloramphenicol only. Plasmids prepared from these transformants, were electrophoresed, blotted and hybridized with radioactively labelled pMC524/MBM. pMC524/MBM hybridized with each plasmid prepared from chloramphenicol resistance transformed clinical isolates indicating strong DNA homology of pMC524/MBM with the plasmid present in the transformed strains. The MIC for chloramphenicol for these transformants was determined and found to be 125 μg/ml. The value was same as that of the original plasmid donor strain *S. aureus* MC524. pMC524/MBM was found to be stable in its original host *S. aureus* MC524 in the presence of chloramphenicol. The plasmid found in all the transformed clinical strains of *S. aureus* also need chloramphenicol for its stable maintenance.

**Discussion**

The most important means of the spread of antibiotic resistance in major bacterial species is by conjugation through the resistant genes residing on plasmids. Plasmid mediated drug resistance was first demonstrated by Japanese workers in *Shigella*
Inactivation of chloramphenicol by *Escherichia coli* was reported first in 1967. Results obtained in this study suggest that pMC524/MBM, a native staphylococcal plasmid of size 2.889 kb,

bearing a chloramphenicol resistant gene, is capable of transferring and expressing itself, in some clinical strains of *S. aureus* without any alteration during the course of transfer. The MIC values of chloramphenicol for *S. aureus* MC524 and different *S. aureus* transformants indicate that the plasmid is efficiently expressed in *S. aureus* transformants.

The spread of resistance via conjugative (intraspESIES, interspecies and intergeneric) plasmid
of 17 to 20 megadaltons that code for chloramphenicol and other antibiotics was found in Streptococci. Interspecies plasmid transfer between Bacillus subtilis and other Bacillus species was reported from Amherst, Massachusetts in 1987. A 3.2 kb plasmid (WBG1022), encoding chloramphenicol resistance was isolated from S. aureus in Perth, Australia, which could be transferred in mixed culture. Report of co-transfer of a non-conjugative 3.5 kb chloramphenicol resistance plasmid along with a conjugative plasmid pXU10 is found. Spread of methicillin resistance via plasmid, from one strain of S. aureus to another in mixed culture was noticed in 1999 in Poland. In our study, the plasmid isolated was of much smaller size (2.889 kb) than reported by other workers.

Under different conditions with antibiotic selection pressure, the resistant determinants are picked up by different bacteria, specially in the hospital environment, as survival measures and thus becomes an important factor in the dissemination of antibiotic resistance. Our results provide evidence that pMC524/MBM, a native staphylococcal chloramphenicol resistant plasmid, is capable of expressing itself in different S. aureus clinical isolates, without undergoing any modification or alteration during this process of interspecies transfer through transformation. The sequencing of the plasmid revealed that it has a size of 2.889 kb. In most instances reported so far the transfer of plasmid mediated antibiotic resistance is conjugal transfer, but our study reports nonconjugalional transfer of chloramphenicol resistance.

With the strong antibiotic selection pressure in hospital environments, this type of small plasmid probably causes rapid emergence and spread of antibiotic resistance in other hospital pathogens.

Acknowledgment

The authors are grateful to Dr R P Novick, Sknraill Institute, New York, USA, for gift of S. aureus strain RN 4220. Authors thank the Staphylococcal Phage Typing Centre, Malauna Avad Medical College, New Delhi for phage typing of S. aureus strains. Thanks are due to Prof Uma Dasgupta, Department of Molecular Biology and Biophysics, University of Calcutta, Kolkata for radioactive hybridization studies. The partial financial assistance from the University Grants Commission (UGC) and Council of Scientific and Industrial Research (CSIR), New Delhi, is acknowledged.

References


Reprint requests: Dr Manjusri Bal, Section of Microbiology, Department of Physiology, University College of Science & Technology, University of Calcutta, 92 APC Road, Kolkata 700009, India
Extended spectrum beta-lactamase production in clinical isolates from hospitals at Kolkata

Suranjana Arora (Ray) and Manjusri Bal*
Department of Physiology, University College of Science & Technology, 92, A P.C Road, Kolkata 700 009, India

Clinical isolates resistant to beta-lactam antibiotics have become an ever-increasing problem, especially due to the production of Extended-Spectrum beta-lactamases (ESBLs) that can hydrolyze a wide spectrum of beta-lactam antibiotics. The present study was undertaken to assess the prevalence of ESBL producers in major hospitals of Kolkata. 284 non-repeat clinical isolates were taken from five major hospitals of Kolkata and screened for ESBL production by Disk Agar Diffusion (DAD) using third generation cephalosporins (3GC) and Double Disk Synergy Test (DDST) with and without clavulanic acid (CA), as per National Committee for Clinical Laboratory Standards (NCCLS). 87 (30.6%) strains were resistant to at least two 3GC out of which 46 (16.2%) were found to be ESBL-producers and confirmed phenotypically by DDST. We found ESBL production in 26 (56.5%) of Escherichia coli, 12 (26.1%) of Klebsiella pneumoniae, 4 (8.6%) of Klebsiella spp., 2 (4.3%) of Pseudomonas aeruginosa, 2 (4.3%) of Proteus vulgaris. Some of the representative isolates were screened for the presence of plasmid DNA. Both large and small plasmids were found in these strains. We report a finding of 16.2% of ESBL producing clinical strains from Kolkata. ESBL producers can cause a major therapeutic failure in Kolkata if not detected and reported in time.

Key Words: Clinical isolates, beta-lactam antibiotics, clavulanic acid, ESBL producers.

Dramatic increase in bacterial resistance to beta-lactam antibiotics during the last decade has been the cause of concern. Beta-lactams inhibit enzymes involved in the synthesis of bacterial cell wall endangering their survival. A common mechanism of bacterial resistance to beta-lactam antibiotics is the production of beta-lactamase enzymes that cleave the structural beta-lactam ring of penicillin group of drugs. More than 60 different types of beta-lactamases have been described from Gram-negative and Gram-positive organisms.

Classical beta-lactamases such as TEM-1, TEM-2 and SHV-1, that are found frequently in Gram-negative pathogens, can hydrolyze first and second-generation cephalosporins, but not the modern third generation cephalosporins. Extended spectrum beta-lactamases (ESBLs) are spontaneous variants of TEM-1, TEM-2 and SHV-1. They carry single amino acid substitution at the active site enabling them to extend their substrate spectrum towards the third generation cephalosporins like Cefotaxime and Cefpodoxime. The term ESBL refers to beta-lactamase enzymes produced mainly by Klebsiella spp. and E. coli, which encode for resistance to broad-spectrum beta-lactam antibiotics that normally have activity against Gram-negative bacilli. The resulting resistance is of great clinical concern. Beta-lactamase inhibitors, such as clavulanic acid and sulbactam, can block these enzymes.

Material & Methods

Two hundred and eighty four different non-repeat pathogenic strains were collected from five city hospitals namely Calcutta Medical College (CMC), Nilratan Sirkar Medical College (NRS), Seth Sukhlal Karnan Medical (S.S.K.M.) Hospital, School of Tropical Medicine (STM) and R.G.Kar Medical College and Hospital (RGK). The bacteria were isolated, 90 (31.7%) from pus, 132 (46.5%) urine, 57 (20.1%) sputum and 5 (1.8%) others. The above samples were collected from admitted patients during a period of 15 months from February 2002 to April 2003. The strains were maintained on Nutrient Agar slants and sub-cultured every month. They were also preserved in nutrient broth with glycerol at -70°C. The identity of the strains was confirmed using the biochemical tests like citrate, indole, triple sugar iron agar, urease and lactose.

The isolates were examined for their response to
Table 1. Antibiotic resistance pattern of Extended Spectrum Beta-Lactamase (ESBL) production.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antibiotic Resistance</th>
<th>Organism</th>
<th>Antibiotic Resistance</th>
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<tr>
<td>K. pneumoniae</td>
<td>CMC-70 **A,Am,Cpl,Ci, Co,E,G,T, Cep,Ci</td>
<td>E. coli</td>
<td>CMC-76 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>K. pneumoniae</td>
<td>CMC-40 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Ci</td>
<td>E. coli</td>
<td>CMC-72 **A,Am,Cpl,Ci, Co,E,G,T, Cep,Ci, Ao</td>
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<td>K. pneumoniae</td>
<td>CMC-99 **A,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>STM-19 **A,Am,Cpl,Ci, Co,E,G,T, Cep,Ci, Ao</td>
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<td>K. pneumoniae</td>
<td>CMC-19 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>STM-9 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>K. pneumoniae</td>
<td>CMC-56 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>CMC-1 **A,Am,Cpl,Ci, Co,E,G,T, Ce,Cep,Ci</td>
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<tr>
<td>K. pneumoniae</td>
<td>CMC-1 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>CMC-35 **A,Am,Cpl,Ci, Co,E,G,T, Ce,Cep,Ci</td>
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<td>K. pneumoniae</td>
<td>CMC-72 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>CMC-20 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>K. pneumoniae</td>
<td>CMC-7 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>NRS-7 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>K. pneumoniae</td>
<td>CMC-72 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>CMC-59 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>K. pneumoniae</td>
<td>NRS-104 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>CMC-64 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>K. pneumoniae</td>
<td>NRS-169 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>CMC-37 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>K. pneumoniae</td>
<td>NRS-105 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>CMC-33 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>Klebsiella spp.</td>
<td>NRS-110 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>NRS-73 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>Klebsiella spp.</td>
<td>CMC-109 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>NRS-31 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>Klebsiella spp.</td>
<td>NRS-84 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
<td>E. coli</td>
<td>NRS-81 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>Klebsiella spp.</td>
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<td>E. coli</td>
<td>CMC-71 **A,Am,Cpl,Ci, Co,E,G,T, Ca,Ce,Cep,Ci, Ao</td>
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<td>P. aeruginosa</td>
<td>CMC-2 **A,Am,Cpl,Ci, Co,E,G,T, Cep,Ci</td>
<td>E. coli</td>
<td>NRS-45 **A,Am,Cpl,Ci, Co,E,G,T, Cep,Ci</td>
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<tr>
<td>P. aeruginosa</td>
<td>NRS-97 **A,Am,Cpl,Ci, Co,E,G,T, Cep,Ci</td>
<td>E. coli</td>
<td>NRS-101 **A,Am,Cpl,Ci, Co,E, Ca,Ce,Cep</td>
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<tr>
<td>P. vulgaris</td>
<td>NRS-14 **A,Am,Cpl,Ci, Co,E,G,T, Cep,Ci</td>
<td>E. coli</td>
<td>CMC-66 **A,Am,Cpl, Co,E,G,T, Cep,Ci</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>CMC-76 **A,Am,Cpl,Ci, Co,E,G,T, Cep,Ci</td>
<td>E. coli</td>
<td>NRS-89 **A,Am,Cpl,Ci, Co,E,G,T, Cep,Ci</td>
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<td>E. coli</td>
<td>STM-11 **A,Am,Cpl,Co,E,G,T, Cep,Ci, Ca,Ce,Cep,Ci</td>
<td>E. coli</td>
<td>NRS-200 **A,Am,Cpl,G,T, Ce,Cep,Ci</td>
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<td>E. coli</td>
<td>STM-12 **A,Am,Cpl,Ci, Co,E,G,T, Cep,Ci, Ca,Ce,Cep,Ci</td>
<td>E. coli</td>
<td>CMC-83 **A,Am,Cpl,G,T, Ce,Cep,Ci</td>
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<td>E. coli</td>
<td>STM-5 **A,Am,Cpl,Ci, Co,E,G,T, Cep,Ci, Ca,Ce,Cep,Ci</td>
<td>E. coli</td>
<td>CMC-40 **A,Am, Co,E,G,T, Ca,Ce,Cep,Ci</td>
</tr>
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</table>

** A- Ampicillin (10μg), Am-Ampicillin (20μg), Cep-Cephalaxin (30μg), CI- Ciprofloxacin (5μg), Co- Cotrimaxazole (sulphamethazol/trimethoprim 1.25/23.75μg), E- Erythromycin (10μg), G- Gentamycin (10μg), T- Tetracycline (30μg), Ca- Ceftazidime (30μg), Cep- Cefotaxime (30μg), Cep- Cefpodoxime (10μg), Ci- Ceftrizone (30μg), Ao- Aztreonam (10μg). All are sensitive to Am/CA- Amoxycillin/Clavulanic acid (20/10μg)

Fourteen antibiotics (Table 1). All these antibiotic solutions were prepared in sterile water except erythromycin. Erythromycin was first dissolved in alcohol and diluted with sterile water. For Disc Agar Diffusion (DAD) and Double Disc Synergy Test (DDST) Muller Hinton Broth and agaragar were used.

**Disc agar diffusion method (DAD):** The test bacterium, taken from an over-night culture was freshly grown for 4h in Muller Hinton Broth. With this culture a bacterial lawn was prepared on Muller Hinton Agar. Filter paper discs of 6 mm size soaked in antibiotic solutions were used to find out antibiotic susceptibility pattern for fourteen antibiotics. The zone of bacterial growth inhibition surrounding the disc was measured and compared with a standard for each drug. This gave a profile of drug susceptibility vis-à-vis antibiotic resistance4.
Double disc synergy test (DDST): For detecting suspected ESBL producing bacteria, NCCLS recommendation was used. For detection of ESBL’s in Klebsiella spp. and E. coli, NCCLS currently recommends an initial screening test with any two of the following β-lactam antibiotics: cefpodoxime, cefazidime, aztreonam, cefotaxime or ceftriaxone. Isolates resistant to at least two of the above 3GC are ESBL producers. DDST was done on these bacteria. In DDST, synergy was determined between a disc of cefotaxime (30 μg) and a disc of cefotaxime plus CA (30 μg and 10 μg) which were placed at a distance of 20 mm apart on a lawn of culture of the suspected ESBL producing clinical isolates on Mueller Hinton Agar (MHA). Discs containing CA were prepared by applying 10 μL of a 1000 μg mL⁻¹ CA stock solution to each disk. The test organism was considered to produce ESBL if the zone size around the third generation antibiotic plus clavulanic acid increased >5 mm in comparison to the third generation cephalosporin disc alone. This increase occurs because the ESBL produced by the isolates are inactivated by clavulanic acid.

Nitrocefin spot test: The crude enzyme was prepared by centrifuging overnight culture of the organisms at 10,000 r.p.m at 4°C for 10 minutes. The pellets were then sonicated at 8 μm (in Soniprep, U.K) for 15 sec (two cycles) with 10 secs cooling in between sonications. The sonicated material was centrifuged again to obtain the enzyme. 10 μL of enzyme was incubated with 50 μL of Nitrocefin (1.5 mM, Calbiochem, Germany) in the well of a microtitre plate for 30 min. at room temperature. The presence of beta-lactamase was detected if color of Nitrocefin (working solution prepared according to manufacturer’s instruction) changed from yellow to pinkish-orange.

Microiodometric determination of β-lactamase activity: Microiodometric determination was done according to the method of Sykes and Nordstrom using benzyl-penicillin as substrate.

Plasmid isolation: Plasmid DNA was prepared by the method of Kado and Liu. DNA preparation was preserved in TAE buffer (composition per L): 48.4 g Tris, 11.2 g, Glacial acetic acid, 20 mL EDTA from 0.5 M stock solution]. pH 8.5. Electrophoretic run was carried out at 100V cm⁻¹ for 4 h in the same buffer. The gel was stained with ethidium bromide (0.5 μg mL⁻¹).

Results and Discussion
Among the forty six strains found to be extended spectrnum beta-lactamase (ESBL) producers, maximum resistance was found against ampicillin (100%) followed by amoxycillin (93.5%) and cephalaxin (69%) (Table 1). Among the third generation cephalosporins (3GC), cefpodoxime (86%) showed the maximum resistance rather than ceftazidime (71.7%) (Table 1) although world wide more nosocomial emergence of Ceftazidime resistance has been reported. Of 284 strains tested, 87 isolates were resistant to at least two 3GC and showed positive nitrocefin spot test. Among these 87 isolates, 46 (16.2%) were found to be extended spectrum beta-lactamase (ESBL) producers, as shown by the enhancement of zone inhibition by CA on performing DDST (Figure 1). We observed that the zone size around the third generation cephalosporins disc plus clavulanic acid increased >5 mm in comparison to the third generation cephalosporin disc alone in DDST (Table 2). We found maximum extended-spectrum beta-lactamases production in E.coli (86.5%), followed by Klebsiella pneumoniae (26.1%), then Klebsiella spp., Enterobacter aerogenes (8.6%), Pseudomonas aeruginosa (4.3%) and Proteus vulgaris (4.3%). Thus we report a finding of 16.2% of ESBL producers from Kolkata hospitals.
strains was also tested using benzyl penicillin and cefotaxime as substrates following the Microiodometric assay method of Sykes and Nordstrom. The specific activity of beta-lactamase enzyme varied with strain but all the strains tested positive for beta-lactamase production. Hence by both the methods the beta-lactamase production was found to be positive (Table 3).

Since the ESBL genes are typically located on plasmids and are readily transferable among species', we examined four *E.coli* and three *Klebsiella pneumoniae*, ESBL producing isolates for the presence of plasmid DNA. These strains showed the presence of a large plasmid > 53.4 kb and small plasmids of 6 kb to less than 2 kb. *E.coli* V517 was used as reference strain (Fig. 2). It is reported that genes that code ESBLs production are located on large conjugative plasmids of sizes 80 kb to 160 kb but we found the maximum size to be just greater that 53.4 kb. We, as yet could not confirm that the plasmids found in these strains were responsible for beta-lactamase production.

Extended-spectrum beta-lactamase production has been reported from all over the world. Gram negative bacilli producing ESBL were first described in Germany in 1983.

Table 3. Detection of beta-lactamase by Microiodometric method and nitrocefin spot test.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Specific BP*</th>
<th>Activity BP*</th>
<th>Nitrocefin Spot Test</th>
<th>Organism</th>
<th>Specific Ce**</th>
<th>Activity Ce**</th>
<th>Nitrocefin Spot Test</th>
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<td><em>E. coli</em> STM-11</td>
<td>0.1605</td>
<td>0.3215</td>
<td>+</td>
<td><em>E. coli</em> NRS-200</td>
<td>0.5242</td>
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<td>0.3192</td>
<td>0.2581</td>
<td>+</td>
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<td>0.6454</td>
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<td>0.4181</td>
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<td>0.6103</td>
<td>++</td>
<td><em>K. pneumoniae</em> CMC-70</td>
<td>0.0952</td>
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<td><em>E. coli</em> CMC-72</td>
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<td>0.0413</td>
<td>++</td>
<td><em>K. pneumoniae</em> CMC-40</td>
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<tr>
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<td>0.3210</td>
<td>++</td>
<td><em>K. pneumoniae</em> CMC-99</td>
<td>0.0633</td>
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<td>0.5210</td>
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<td>0.1212</td>
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<td><em>K. pneumoniae</em> CMC-56</td>
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<td>0.8392</td>
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<tr>
<td><em>E. coli</em> CMC-35</td>
<td>0.0010</td>
<td>0.0217</td>
<td>+</td>
<td><em>K. pneumoniae</em> CMC-63</td>
<td>0.0810</td>
<td>0.7362</td>
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<tr>
<td><em>E. coli</em> CMC-20</td>
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<td><em>E. coli</em> NRS-7</td>
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<td>0.6638</td>
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<td>0.4398</td>
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<td>0.0373</td>
<td>0.0545</td>
<td>+ + +</td>
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<tr>
<td><em>E. coli</em> NRS-81</td>
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<td>0.7391</td>
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<td><em>E. coli</em> CMC-71</td>
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<td>0.4130</td>
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<td>0.0214</td>
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<td>0.0103</td>
<td>+</td>
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<td>0.0021</td>
<td>0.0610</td>
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<td>0.1361</td>
<td>++</td>
<td><em>P. aeruginosa</em> NRS-97</td>
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<td><em>E. coli</em> CMC-66</td>
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<td>0.0889</td>
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<td><em>P. vulgaris</em> NRS-14</td>
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<td>0.0401</td>
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<td><em>E. coli</em> NRS-89</td>
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<td>0.3030</td>
<td>+</td>
<td><em>P. vulgaris</em> CMC-76</td>
<td>0.0310</td>
<td>0.0310</td>
<td>+</td>
</tr>
</tbody>
</table>

*BP-Benzyl penicillin, **Ce-Cefotaxime


3y 1994, the Center for Disease control and Prevention as well as National Nosocomial Infections Surveillance System (NNIS) reported that 8% of *Klebsiella* spp. produced ESBLs1$. A 1995-96 study in Richmond, Virginia reported 1.5% of isolates that produced ESBLs4$. In United States, a 1990-93 survey of ICUs of 400 hospitals recorded an increase from 3.6% to 14.4% in ESBL producing strains of *Klebsiella* spp.5$. In Europe, in 1995, about 20-25% *Klebsiella* spp in ICUs were found to be ESBL producers, whereas in France it was 30-40%6$.

From 1993 onwards, presence of various types of β-lactamase in different nosocomial species were also reported from several towns and cities of India. In 1995, there were reports from Vellore, of fifteen strains of *K. pneumoniae* producing ESBL7$. In 1997, 25.8% of *K. pneumoniae* having extended-spectrum beta-lactamase mediated resistance to third generation cephalosporins was reported from Nagpur, Central regions of India8$. In 2001, from southern regions of India there was a report of prevalence of extended-spectrum beta-lactamase producing *Klebsiella* spp. causing nosocomial respiratory infection9$. 6.6% of *Klebsiella* spp. were reported to be ESBL producers in Chennai9$. 68% of ESBL producers were reported in 2002 from Delhi10$. ESBL was detected in 86.6% of *Klebsiella* spp., 73.4% of *Enterobacter* spp., and 63.6% of *E. coli* strains in a study done on neonatal septicemia from Lucknow11$. We present here the first report of extended-spectrum beta-lactamase producing (16.2%) from Kolkata, India. We also found ESBL production in bacterial strains other than *E. coli* and *Klebsiella pneumoniae*. Therapeutic options available in serious infections may become even more restricted, especially if the ESBL producing bacterial infections becomes widespread in Kolkata.

Early detection of emerging trends in antimicrobial resistance may facilitate implementation of effective control measures. The laboratory testing of antibiotic susceptibility contributes directly to patient care and expertise of the microbiology laboratory can have a powerful influence on antibiotic usage preventing the emergence of antimicrobial drug resistance.

**Acknowledgement**

The authors are grateful to Dr. P.C. Banerjee, Deputy Director, Indian Institute of Chemical Biology, Kolkata for help in experimental design and Dr. Amit Pal, Dept. of Pathophysiology, National Institute of Cholera and Enteric Diseases, Kolkata for helping with plasmid isolation. We thank Ranbaxy Pharmaceuticals for gifting the Imipenem disks and Glaxo-Smith Kline for providing Clavulanic acid.

**References**


Received 27 November 2004, final revision May 2005 and accepted June 2005.
AmpC β-lactamase producing bacterial isolates from Kolkata hospital

Suranjana Arora & Manjusri Bal

Section of Microbiology, Department of Physiology, University of Calcutta, University College of Science & Technology, Kolkata, India

Received June 4, 2004

Background & objectives: The widespread use of β-lactam antibiotics has lead to the development of resistance to this group of antibiotics in bacterial pathogens due to β-lactamase production. Information on such pathogens is not available from eastern region of India. This study was undertaken to determine the AmpC β-lactamase production in pathogens isolated from hospitalized patients in Kolkata.

Methods: Non-repeat clinical isolates (254) from pus, urine, sputum and other clinical specimens of hospitalized patients were taken. Disk agar diffusion (DAD) and minimum inhibitory concentration (MIC) with different β-lactam antibiotics, and double disc synergy test (DDST) with clavulanic acid and sulbactam were done. Disk antagonism test (DAT) and three-dimensional extract test (TDET) were conducted for phenotypic confirmation of AmpC and inducible AmpC β-lactamase production. Nitrocefin spot test and microlodometric assay of β-lactamase were also performed.

Results: Twenty seven isolates were found to be resistant to cefoxitin, a α-methoxy-β-lactam. Of these, 19 were observed to be AmpC β-lactamase producers and 4 were inducible AmpC β-lactamase producers by DDST, DAT and TDET. Remaining 4 were non AmpC β-lactamase producers. Of the 23 AmpC β-lactamase producers, the distribution of different species was as follows: Escherichia coli 11 (47.8%), Pseudomonas aeruginosa 4 (17.3%) Klebsiella pneumoniae 3 (13%), and Klebsiella aeruginosa 1 (4.3%).

Interpretation & conclusion: Our finding showed 6.7 per cent AmpC β-lactamase and 1.4 per cent inducible AmpC β-lactamase producing clinical isolates from Kolkata. AmpC β-lactamase producing bacterial pathogens may cause a major therapeutic failure if not detected and reported in time.

Key words: AmpC β-lactamase - cefoxitin - clavulanic acid - inducible AmpC β-lactamase - sulbactam - three-dimensional extract test (TDET)

A common mechanism of bacterial resistance to β-lactam antibiotics is the production of β-lactamase enzymes that cleave the structural β-lactam ring of these drugs. This is the predominant mechanism of β-lactam resistance in Gram-negative bacteria. Over the last two decades many new β-lactams have been
developed that were specifically designed to be resistant to hydrolytic actions of β-lactamase. However, with this new class of drug that has been used to treat patients, new types of β-lactamases emerged. AmpC β-lactamase is one of these new types of β-lactamases.

AmpC β-lactamases are cephalosporinases, which belong to the molecular class C as classified by Ambler in 1980 and Group I under a classification scheme of Bush et al. AmpC β-lactamases are more sensitive to inhibition by sulbactam than by clavulanate or tazobactam. These are clinically significant as they may confer resistance to a wide variety of β-lactam drugs, including α-methoxy-β-lactams, narrow, expanded and broad-spectrum cephalosporins, aztreonam, a monobactam and most significantly β-lactam plus β-lactamase inhibitor combinations (viz., ampicillin-clavulanic acid, pipericillin-tazobactam, etc.).

In many species, β-lactamases are normally produced at very low levels but are induced to several hundred fold higher by the presence of β-lactams (viz., cefoxitin, cefotaxime, etc.) and certain β-lactam inhibitors (viz., clavulanic acid). Inducible AmpC β-lactamases are such examples. Amoxicillin-clavulanic acid combination is commonly used in controlling β-lactamase producing pathogens, as clavulanic acid acts as an inhibitor to many β-lactamases. But in case of inducible AmpC β-lactamases, this type of drug can cause more harm than help.

β-lactamase producing bacteria can cause serious therapeutic failure if not detected on time. Though the clinicians treat infections based on the results of antibiotic susceptibility tests available, the number of infections caused by AmpC β-lactamase producing organisms is on the rise and pose a threat to the patients due to treatment failure. A few groups have reported the occurrence of β-lactamases producing bacteria from northern and southern regions, but there are not much data available on these pathogens from Kolkata or anywhere in the eastern region. The present study was therefore undertaken to find out the presence of AmpC and inducible AmpC type of β-lactamases producing clinical isolates from hospitalized patients in Kolkata using standard methods presently available for their detection. It may be mentioned here that currently there is no clear consensus regarding guidelines for performing tests for the phenotypic screening or confirmatory tests for the isolates that harbour AmpC β-lactamases.

### Material & Methods

**Bacterial isolates:** A total of 284 non-repeat clinical isolates collected from patients admitted to various wards during February 2002 to April 2003 (90 (31.7%) from pus, 132 (46.5%) from urine, 57 (20.1%) from sputum, 5 (1.8%) from other specimens such as burns, catheter, throat swab, ear discharge, gastric lavage fluid, and peritoneal fluid) from five hospitals in Kolkata, namely Calcutta Medical College and Hospital (CMC), Nil Ratan Sarkar Medical College (NRS), Seth Sukhla Karnani Memorial Hospital (SSKM), R.G. Kar Medical College (RGK) and School of Tropical Medicine (STM) were included in this study.

**Media and chemicals:** Ampicillin (A, Lyka Labs, India), amoxycillin (Am, Wyeth Lederle Ltd, India), amoxicillin/clavulanic acid (Am/CA, Ranbaxy Laboratories, India) aztreonam (Ao, Hi-media, Mumbai), cefactam (Cfs, Aurobindo Pharma Ltd., India), cefotaxime (Ce, Alkem Laboratories Ltd, India), cefpodoxime (Cep, Universal Medicare Pvt, India), ceftriazone (Ci, Wockhardt, India), cefazidime (Ca, Glaxo, India), cefoxitin (Cn, Hi-media, Mumbai), cefpirome (Cpm, Alkem, India), cephalexin (CpI, Glaxo, India), ciprofloxacin (Cf, Ranbaxy, India), clavulanic acid (CA, Glaxo Smith Kline, UK), co-trimoxazole (Co, sulphamethazine/trimethoprim), Welcome, India, gentamicin (G, Nicolás Piramal, India), imipenem (IPM, Ranbaxy Laboratories, India), piperacillin/tazobactam (Pt, Hi-media, Mumbai), tetracycline (T, Hoeest, India), were used in this study. Antibiotic solutions were prepared in sterile water. For disc agar diffusion (DAD), minimum inhibitory concentration (MIC) and double disk synergy test (DDST) Muller-Hinton broth (MHB, Hi-media, Mumbai) and agar agar (Qualigen Fine Chemicals, India) were used.

**Disc agar diffusion method (DAD):** The test bacterium, taken from an over-night culture (inoculated from a single colony) was freshly grown for 4 h and with this culture a bacterial lawn was prepared on MHA plate. Filter paper disks of 6 mm
size were used to find out antibiotic susceptibility pattern against 10 antibiotics (concentration in µg) [A (10), Am (20), Ao (30), Cf (5), Cn (30), Co (sulphamethazone/trimethoprim, 1.25/23.75), G (10), T (30), Cpl (30) and IPM (10)], four third generation cephalosporins [Ca (30), Ci (30), Ce (30), Cep (10)], one fourth generation cephalosporin [Cpm (30)], three β-lactam+β-lactamase inhibitor combination, viz., amoxicillin-clavulanic acid combination [Am/CA, (20/10)], pipericillin-tazobactam combination [Pt, (100/10)] and cefaperazone-sulbactam [Cfs (75/30)] combination following Kirby-Bauer method. The disks were prepared according to manufacturer's instruction. Strains resistant to cefoxitin (zone diameter less than 18 mm) were suspected to be AmpC β-lactamase producers.

Minimum inhibitory concentration (MIC): The MIC of the antibiotics was determined by two-fold serial broth dilution method.

Double disk synergy test (DDST): In DDST, synergy was determined between a disk of cefotaxime (30 µg) and a disk of cefotaxime plus CA (30 µg + 10 µg) which were placed at a distance of 20 mm apart on a lawn of culture of the suspected β-lactamase producing clinical isolates on MHA. Disks containing clavulanic acid were prepared by applying 10 µl of a 1000 µg/ml clavulanic acid stock solution to each disk. The test organism was considered to produce β-lactamase if the zone size around the cefotaxime plus clavulanic acid increased >5 mm in comparison to the third generation cephalosporin (Ce) disk alone. This increase occurred because the β-lactamases produced by the isolates were inactivated by clavulanic acid. The NCCLS guidelines do not have any standard tests for isolates, that produce AmpC β-lactamase. If the increase in zone size was ≤5 mm or there was no increase in zone size after addition of clavulanic acid in DDST, then the DDST was done with cefotaxime and cefactam (cefperazone/sulbactam, Cfs, 75/30 µg). Here cefactam was used as a source of sulbactam. If the zone of inhibition increased on using sulbactam, the isolates were considered to be AmpC β-lactamase producers.

Disk antagonism test (DAT): The disk antagonism test was used to detect the inducibility of β-lactamase. Disks of inducing agent cefoxitin (Cn) and cephalosporins (Cpm, Ca, Ci and Ce) were placed on the surface of the test bacterial lawn on MHA plates on a lawn of bacterial culture of the suspected inducible AmpC β-lactamase producers separated by 15 mm. The plates were examined after overnight incubation at 37°C. If blunting of the cephalosporin disks adjacent to the cefoxitin disks occurred, the organisms were considered to produce inducible AmpC β-lactamase.

Three-dimensional extract test (TDET): 50 µl of a 0.5 McFarland bacterial suspension prepared from an overnight MHB was inoculated into 12 ml of MHB and the culture was grown for 4 h at 35°C. The cells were concentrated by centrifugation, and crude enzyme preparations were made by sonicating the pellets at 8 µm (in Soniprep, UK) for 15 sec (two cycles) with 10 sec cooling in between sonications. The NCCLS guidelines do not have any standard tests for isolates, that produce AmpC β-lactamase. If the increase in zone size was <5 mm or there was no increase in zone size after addition of clavulanic acid in DDST, then the DDST was done with cefotaxime and cefactam (cefperazone/sulbactam, Cfs, 75/30 µg). Here cefactam was used as a source of sulbactam. If the zone of inhibition increased on using sulbactam, the isolates were considered to be AmpC β-lactamase producers.

Nitrocefin spot test: The crude enzyme was prepared by centrifuging overnight culture of the organisms at 10,000 r.p.m at 4°C for 10 min. The pellets were then sonicated at 8 µm (in Soniprep, UK) for 15 sec (two cycles) with 10 sec cooling in between sonications. The sonicated material was centrifuged again to obtain the enzyme; 10 µl of enzyme was incubated with 50 µl of 1.5 mM nitrocefin (Calbiochem, San Diego, USA; working solution prepared according to the manufacturer's instruction) in the well of a microtitre plate for 30 min at room temperature. The presence of β-lactamase was detected if colour of nitrocefin changed from yellow to reddish-orange.

Microiodometric determination of β-lactamase activity: Microiodometric determination was done
Induction of AmpC β-lactamase by clavulanic acid:
For testing, induction of AmpC β-lactamase by clavulanic acid, 10 μg/ml of clavulanic acid was added to MHB in which the test bacteria were grown overnight at 37°C in a gyratory shaker. Enzyme was prepared from this overnight growth as described previously. The enzymes thus obtained were tested by nitrocefin spot test and microiodometric determination of β-lactamase activity.

Isoelectric focusing: Analytical isoelectric focusing was performed with some of the β-lactamase extracts, by the method of Mathew et al. The ampholine range used were pH 3.5-10. The samples were loaded on the gel and given a pre-run of 20 min at 15 mA at constant volts of 1 Watt and then run for 60 min at 50 mA on LKB Multiphor II isoelectric focusing apparatus (Pharmacia, Sweden). β-lactamases with known isoelectric point (pl) were focused as control: 15 μl of TEM 1 (pi 5.4) obtained from E. coli J53 RI (Dr Reddy’s Laboratory, Hyderabad) and 10 μl of SHV-18 (pi 7.8) obtained from Klebsiella pneumoniae ATCC 700603 (Dr Reddy’s Laboratory, Hyderabad) were used. Gels were stained with 10⁻⁴ M nitrocefin. The pl of the enzyme was indicated by a reddish-orange band on the gel bed at a specific position.

Results

Of the 284 non-repeat clinical isolates tested by DAD, 27 (9.5%) were resistant to cefoxitin which was used as a primary selection criterion of AmpC β-lactamases. MIC as well as DDST done on these 27 isolates with Ce, Ce/CA and Cfs showed that 19 (12 from urine, 3 from pus and 4 from sputum and throat swabs) were resistant to inhibition by clavulanic acid but were inhibited by sulbactam (Table I, Fig.1) and tazobactam. Four isolates showed decreased zone of inhibition with clavulanic acid suggesting production of β-lactamases induced by clavulanic acid (Table II). Of the 27 isolates, the remaining four were non-producers of AmpC β-lactamase. All the 23 isolates, which produced AmpC β-lactamase, exhibited high level of resistance to the antibiotics tested by DAD. Seventeen of the AmpC β-lactamase producers were resistant to ciprofloxacin. Ceftazidime showed sensitivity in 9 (39%) of the 23 isolates tested positive for AmpC β-lactamase production. Cefpodoxime resistance was seen in all 23 isolates.

AmpC β-lactamase production was confirmed by performing TDET. All the suspected 19 isolates were found to be positive for TDET; i.e., there was growth along the slit within the zone of inhibition of cefoxitin (Fig. 2). Inducibility of the β-lactamases was further recognized by DAT, which demonstrated blunting of specific cephalosporin (cefotaxime) disks adjacent to the cefoxitin disks (Fig. 3).

AmpC β-lactamase production (including inducible AmpC β-lactamases) was seen in 23 (8.1%) isolates. AmpC β-lactamases were produced by E. coli 11 (47.8%), P. aeruginosa 4 (17.3%), K. pneumoniae 3 (13%) and K. aeruginosa 1 (4.3%). Of the inducible AmpC β-lactamase producing isolates, 3 were isolated from urine samples (K. pneumoniae CMC-40, Proteus vulgaris CMC-90 and P. aeruginosa NRS-3) and 1 from burn patient (P. aeruginosa NRS-226) (Tables I, II).

All AmpC β-lactamase producers were found to be positive by nitrocefin spot test. β-lactamase activity from all the 23 isolates were also tested using benzylpenicillin and cefotaxime as substrates following the microiodometric assay. The specific activity of β-lactamase enzyme varied from one organism to another but all were positive for β-lactamase production. Four isolates were found to be producing inducible AmpC β-lactamase which was also confirmed enzymatically. Addition of clavulanic acid in the growth medium induced the production of inducible AmpC β-lactamase in case of both the substrates, benzylpenicillin and cefotaxime, from 1.1 fold to 16 fold, confirming that clavulanic acid is an inducer of this enzyme in vivo (Table III).

Isoelectric focusing showed that both TEM-1 (pl 5.4) and SHV-18 (pl 7.8) type of β-lactamase enzymes were the predominant types of enzyme present in the AmpC β-lactamases (Figs 4, 5). In case of some samples including the reference strain E. coli J53RI, a band is seen at the point of application (Figs 4, 5); this may be due to aggregation of proteins which did not migrate under the experimental conditions applied.
Table I. Clinical isolates showing AmpC β-lactamase production

<table>
<thead>
<tr>
<th>Clinical isolate</th>
<th>Source of sample</th>
<th>DAD (resistant to)</th>
<th>MIC (µg/ml)</th>
<th>DDST (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ce*</td>
<td>C/S*</td>
<td>Ce*</td>
</tr>
<tr>
<td><em>Escherichia coli</em> STM-70</td>
<td>Urine</td>
<td>A,Am,Cpl,Cf , Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA</td>
<td>128</td>
<td>16</td>
</tr>
<tr>
<td>E. coli RT-11</td>
<td>Catheter</td>
<td>A,Am,Cpl,Cf,Co,G,T,Cep,Ci, Ce, Ao, Am/CA</td>
<td>128</td>
<td>16</td>
</tr>
<tr>
<td>E. coli NRS-55</td>
<td>Urine</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA</td>
<td>128</td>
<td>8</td>
</tr>
<tr>
<td>E. coli CMC-67</td>
<td>Pus</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ce,Cep,Ci, Ao, Am/CA,</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td>E. coli CMC-68</td>
<td>Urine</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA</td>
<td>128</td>
<td>16</td>
</tr>
<tr>
<td>E. coli CMC-28</td>
<td>Urine</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td>E. coli NRS-29</td>
<td>Urine</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep, Ao, Am/CA,</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td>E. coli CMC-100</td>
<td>Urine</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA,</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td>E. coli RGK-2</td>
<td>Urine</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td>E. coli STM-9</td>
<td>Urine</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td>E. coli STM-4</td>
<td>Urine</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA</td>
<td>&gt;256</td>
<td>8</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> STM-107</td>
<td>Throat swab</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td>K. pneumoniae CMC-10</td>
<td>Sputum</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td>K. pneumoniae CMC-242</td>
<td>Sputum</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td>K. pneumoniae NRS-83</td>
<td>Throat swab</td>
<td>A,Am,Cpl, T,Cep, Ao, Am/CA</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td><em>K. aerogenes</em> NRS-26</td>
<td>Urine</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Cep,Ci, Ao, Am/CA</td>
<td>128</td>
<td>8</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa CMC-3</td>
<td>Burns</td>
<td>A,Am,Cpl, G,T,Ca,Ce,Cep, Ao, Am/CA</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> SSKM_1</td>
<td>Burns</td>
<td>A,Am,Cpl,Cf,Co,G,T,Cep,Ci, Ao, Am/CA</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> NRS-264</td>
<td>Urine</td>
<td>A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA</td>
<td>&gt;256</td>
<td>8</td>
</tr>
</tbody>
</table>

A, Ampicillin; Am, amoxicillin; Cpl, cephalaxin; Cf, ciprofloxacin; Co, cotrimoxazole; G, gentamycin; T, tetracycline; Ca, ceftazidime; Ce, cefotaxime; Cep, cefpodoxime; Ci, ceftarizone; Cpm, cefpirome; IPM, imipenem; Am/CA, amoxicillin/clavulanic acid; C/S, ceferazone/subactam; Pt, pipericillin/tazobactam (all the above isolates were sensitive to Pt, pipericillin/tazobactam; Cpm, cefpirome and IPM, imipenem)

*Ce, Cefotaxime added 30 µg/disc; **CA, clavulanic acid added in constant amount of 10 µg/disc for DAD and DDST, 4 µg/ml for MIC

DAD, Disc agar diffusion; DDST, double disc synergy test; MIC, minimum inhibitory concentration
Fig. 1. Double disk synergy test done on *E. coli* STM 70; C, cefotaxime disk; CA, cefotaxime + clavulanic acid disk; S, cefactum (ceperazone + sulbactam) disk; CA with C shows no synergy, whereas C with S shows enhanced zone of inhibition indicating production of AmpC β-lactamase.

### Table II. Clinical isolates showing inducible AmpC beta-lactamases production

<table>
<thead>
<tr>
<th>Clinical isolates</th>
<th>DAD (Resistant to)</th>
<th>Source of MIC (µg/ml)</th>
<th>DDST (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Klebsiella pneumoniae</em> CMC-40</td>
<td>A, Am, Cpl, C, G, T, Ca, Ce, Cep, Ci, Cn, Ao, Am/CA</td>
<td>Urine</td>
<td>32</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> NRS-3</td>
<td>A, Am, Cpl, C, G, T, Ca, Ce, Cep, Ci, Cn, Ao, Am/CA</td>
<td>Urine</td>
<td>64</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> NRS-226</td>
<td>A, Am, Cpl, C, G, T, Ca, Ce, Cep, Ci, Cn, Ao, Am/CA</td>
<td>Burns</td>
<td>08</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em> CMC-90</td>
<td>A, Am, Cpl, C, G, T, Ca, Ce, Cep, Ci, Cn, Ao, Am/CA</td>
<td>Urine</td>
<td>128</td>
</tr>
</tbody>
</table>

*C, Cefotaxime added 30 µg/disc; **CA, clavulanic acid added in constant amount of 10 µg/disc for DAD and DDST and 4 µg/ml for MIC.

A, Ampicillin; Am, amoxicillin; Cpl, cephalaxin; C, ciprofloxacin; Co, cotrimoxazole; G, gentamycin; T, tetracycline; Ca, cefazidime; Ce, cefotaxime; Cep, cefpodoxime; Ci, ceftriazone; Am/CA, amoxycillin/clavulanic acid; Cfs, cefperazone/sulbactam (all the above strains very sensitive to Pt, pipericillin/tazobactam; Cpm, cefpirome and IPM, imipem)

DAD, Disc agar diffusion; MIC, minimum inhibitory concentration; DDST, double disc synergy test

### Table III. Specific activity of the clinical isolates producing inducible AmpC β-lactamase by microiodometric methods in the presence and absence of clavulanic acid

<table>
<thead>
<tr>
<th>Clinical isolate</th>
<th>Benzylpenicillin as substrate</th>
<th>Cefotaxime as substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without CA*</td>
<td>With CA*</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> CMC-40</td>
<td>0.014</td>
<td>0.0479</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> NRS-3</td>
<td>0.0094</td>
<td>0.1509</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> NRS-226</td>
<td>0.0471</td>
<td>0.0735</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em> CMC-90</td>
<td>0.0868</td>
<td>0.0981</td>
</tr>
</tbody>
</table>

*CA, Clavulnic acid added 10 µg/ml in vivo
**Fig. 2.** Three dimensional extraction test. The disk placed in the middle is cefoxitin (Cn). One slit was filled with β-lactamase prepared from *E. coli* NRS 55. Regrowth along the slit in the zone of inhibition indicates production of AmpC β-lactamase by *E. coli* NRS 55. The other slit was filled with β-lactamase prepared from *E. coli* CMC 35. No growth was found in the case of *E. coli* CMC 35, a non-AmpC β-lactamase producer, which was used as negative control.

**Fig. 3.** Disk antagonism test done on *E. coli* CMC 100. The disk placed in the middle is cefoxitin (Cn); cefpodoxime (Cp) and ceftriazone (CT) showed no zone of inhibition on the side of cefoxitin (Cn) disk. But cefotaxime (Ce) disk on the right showed blunting of the zone of inhibition on the side of cefoxitin (Cn) disk. This confirms the presence of AmpC β-lactamase in *E. coli* CMC 100.
Discussion

The efficacy of β-lactam group of antibiotics was reduced due to the production of β-lactamases by the resistant bacterial strains. Therefore, search for their inhibitors was initiated to protect the antibiotic activity in vivo against β-lactam resistant pathogens. Clavulanic acid, a naturally occurring β-lactam, had been the first such inhibitor, which is produced by Streptomyces clavuligerus15. Subsequently, a few more viz., sulbactam, a penicillanic acid sulphone16, tazobactam, etc., were found.

Plasmid mediated AmpC β-lactamase from K. pneumoniae isolates was first reported in 1989 from Seoul, South Korea17. Within 1998, nineteen types of plasmid mediated AmpC β-lactamases were reported from Algeria, France, Germany, Greece, India, Pakistan, Taiwan, Turkey, United Kingdom and United States18. The prevalence of AmpC β-lactamase enzyme was 2 per cent in E. coli and 17.1 per cent in K. pneumoniae in China19. Recently, AmpC-type β-lactamase producing K. pneumoniae were also reported from the Republic of Korea20. Plasmid mediated inducible AmpC β-lactamases are still rare.

DHA-1 type of inducible AmpC β-lactamases were first reported from Saudi Arabia in 199821 and later on from Taiwan in 200212. Six of the 51 isolates of Enterobacteriaceae were found to be inducible AmpC β-lactamase producers from Korea22. In Richmond, Virginia, USA, 2.6 per cent of K. pneumoniae were found to be AmpC β-lactamase producers23. Plasmid encoded AmpC type β-lactamases were found in 8.5 per cent of the K. pneumoniae, 6.9 per cent of the K. oxytoca and 4.0 per cent of the E. coli collected from 25 US capital states and the district of Columbia24.

In 2003, 20.7 per cent AmpC enzyme producers were found among Gram-negative bacteria in Guru Tegh Bahadur Hospital, Delhi25. In the same year Subha et al found AmpC β-lactamase production in 24.1 per cent of Klebsiella spp. and 37.5 per cent of E. coli in Chennai26. Shahid et al found 20 per cent of P. aeruginosa producing AmpC β-lactamase in Aligarh27, and in Karnataka, 3.3 per cent of E. coli.
2.2 per cent of *K. pneumoniae*, 5 per cent of *C. freundii*, and 5.5 per cent of *E. aeregenes* (all urinary isolates) were found to harbour AmpC enzymes.

The phenotypic data generated in this study indicated that in Kolkata hospitals (6.7%) of the isolates were phenotypically confirmed to be AmpC β-lactamase producers and 1.4 per cent to be inducible AmpC β-lactamase producers which were less than that reported from Delhi25, Chennai26 and Aligarh27 but more than that was found in Karnataka28.

It has been reported that Amp C β-lactamases had pI values like that of TEM-1, SHV-11 and DHA-1 type of β-lactamases, which are pH 5.4, 7.6, and 7.8, respectively12. In our study, we also found that many of the AmpC β-lactamases are having pI values in the range of pH 5.4 and pH 7.8.

This is perhaps the first report of AmpC β-lactamase and inducible AmpC β-lactamase producing bacteria from Kolkata, India. Clavulanic acid if used as an inhibitor of β-lactamase in the AmpC β-lactamase producing bacteria, can cause therapeutic failure. If the type of β-lactamase produced by the pathogen could be detected along with the antibiogram before administering the β-lactam drug to the patient, therapeutic failure might be avoided. These changes in the bacterial population represent evolutionary upgrades, which provide them a greater potential to resist β-lactam antibiotics and cause formidable therapeutic and diagnostic challenges.

**Acknowledgment**

The authors thank Dr P.C. Banerjee, Deputy Director, Irishian Institute of Chemical Biology, Kolkata for help in experimental design. Dr Dhruvboytik Chattopadhyay, Dean of Science, Department of Biochemistry, UCS&T, Ballygunj, Kolkata, for providing facilities for isoelectric focusing, Dr Magadi Sita Ram, Dr Reddy's Laboratory for providing *K. pneumoniae* ATCC 700603 and *E. coli* 153RI, Dr M K. Mazumdar, Ex-Director, Central Drug Laboratory, Kolkata, for providing *Escherichia coli* ATCC 25922. We thank Ranbaxy Pharmaceuticals for supplying the imipenem disks and Glaxo-Smith Kline for providing clavulanic acid.

**References**


Reprint requests: Dr Manjusri Bal, Section of Microbiology, Department of Physiology, University of Calcutta University College of Science & Technology, 92, A.P.C. Road, Kolkata 700009, India
e-mail: manjusrb@vsnl.net
Expression Of A Staphylococcal Plasmid In Escherichia Coli

Manjusri Bal

Section of Microbiology

Department of Physiology, University of Calcutta, University Colleges of Science & Technology, 92, A.P.C Road, Calcutta 700 009, India

This study was aimed to investigate if any naturally occurring, Gram positive Staphylococcal resident plasmid could replicate and express in a Gram negative host and vice-versa, because such transfer could very well be a clue to the rapid spread of antibiotic resistance among different bacteria. For such study, a multiple antibiotic resistant (Amp, Cm, Km, Sm) clinical strain of Staphylococcus aureus MC524 was chosen, which was found to contain a small plasmid. This plasmid was designated as pMC524/MBM and was studied in detail. It was found that this plasmid could code for chloramphenicol resistance, made of 2889 base pairs, with a single HindIII cutting site. Complete nucleotide sequence of this plasmid is established. pMC524/MBM could be transformed into different strains of S. aureus as well as in Escherichia coli DH5α. The plasmid pMC524/MBM remained unmodified during these transformation processes, as verified by analysing the size, mobility and the restriction enzyme digestion patterns on 0.8% agarose gel — electrophorogram. This fact was further confirmed by Southern blotting of the plasmids isolated from the S. aureus and E. coli transformants followed by hybridisation with radioactively labelled pMC524/MBM. Thus a Cm resistant Staphylococcal resident plasmid can undergo both intra and intergeneric transfer without any modification during the transfer process. Such transfer may be responsible for the rapid dissemination of antibiotic resistance in diverse bacterial genera.

The Emergence Of Citrobacter As A Nosocomial Pathogen

Suranjana Ray* and P. L. Kariholu**

* Dept. of Physiology, Sri. B.M. Patil Med. Coll., Bijapur, Karnataka
** Dept. of Surgery, Sri. B.M. Patil Med. Coll., Bijapur, Karnataka

Nosocomial Infection were studied from October 1999 to July 2000, in a district hospital. The specialties were medicine, surgery, gynae-obstetrics, pediatrics and ICU. The cases studied were 8,530. Out of these 524 patients were found to have nosocomial infection which were confirmed by culture with sensitivity surveillance of wards. The Citrobacter were grown in Conkey and Deoxycholate citrate agar. The species "sandii" was found to be a rapid fermenter of lactose, sacchar positive and indole negative, whereas the "citrobacter diversus" was indole positive and KCN sensitive.

The rate of Citrobacter was unusually high being 70 cases out of the 524 detected with nosocomial infection which is a rate of 13.55% among the pathogens detected. Citrobacter is an uncommon, non-lactose fermenting, Gram-negative enterobacteriaceae which have found to be causing diarrhoea, U.T.I, neonatal brain abscess and meningitis. But in our study we found that both Citrobacter diversus and Citrobacter freundii contributed to wound infection, respiratory tract infection, urinary tract infection and sepsis. Citrobacter is not a common nosocomial pathogen. It is a coliform bacteria commonly found in soil, vegetation, natural water and in contaminated infant formula but though we found coliform bacteria on dressing swabs and hand-wash solution, we could not establish that it was Citrobacter.

The immuno-compromised status of the patients might have contributed for the high incidence or the wide-spread use of broad spectrum antibiotics might have caused it to emerge as a new nosocomial pathogen.
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FEBRUARY 23rd & 24th, 2002
The Changing Face Of Antibiotic Resistance In Hospital Acquired Infection

Suranjana Ray and M.Bal

Microbiology laboratory, Department of Physiology,
University of Calcutta, University College of Science & Technology,
92, A. P. C. Road, Kolkata-700 009

The emergence of antimicrobial resistance strains of pathogenic bacteria has become a great threat to the public health. Right from the discovery of Penicillin as the wonder drug in 1920s- the changing nature of bacterial infection and the continuing development of microbial resistance against it has been challenging mankind. The development and use of antibiotics for chemotherapy of bacterial infection was one of the most remarkable achievements in medicine of the 20th century. However, antibiotic resistant bacteria were found in clinical isolates soon after the introduction of earlier antimicrobial agents into the market. By 1950s most bacteria were producing beta-lactamases. Some of these important organisms are methicillin-resistant Staphylococcus aureus, penicillin-resistant Streptococcus pneumoniae, vancomycin-resistant Enterococcus and certain gram-negative bacilli due to extended-spectrum beta-lactamase production. Of these in our laboratory we have been studying the resistance pattern of *S-aureus* over the last three decades and *Citrobacter* spp, an emerging nosocomial pathogen over the last three years. These resistance to antibiotics which is steadily rising have made microbial therapy extremely difficult. This therapeutic crisis produced by emerging antimicrobial resistance continue to escalate in occurrence leading to the widespread use of empiric combination regimens. The extensive, and often inappropriate use of antibiotics worldwide is the major contributing factor in the emergence and spread of antibiotic resistance.

Chronological Assessment Of Brain Tumor Induction In Rats With Ethyl Nitroso Urea (ENU) And Its Immunotherapeutic Schedule With a novel Biological Response Modifier - The T11TS.

J. Mukherjee, A. Ghosh, S. Sarkar, and Swapna Chaudhuri.

Cellular & Molecular Immunology Unit,
Dept. of Physiology, University College of Medicine,
244 B A.J.C.Bose Road, Kolkata - 700 020.

Brain tumors are a heterogeneous group of neoplasms that can arise from any of the constituent elements of CNS, including neurons, glia, endothelia, and meninges. The most common primary brain tumor in adults is glial neoplasms, this accounts for approximately 65% of all primary CNS tumors. In cancer research experimental animal brain tumor model is very important. Ethyl Nitroso Urea (ENU) long being used as a potent neurocarcinogen and produce tumors with biological and morphological similarities with naturally occurring neural neoplasms in man. In our laboratory, brain tumors were successfully induced after administration of ENU in neonatal rats. Unfortunately, radical treatments such as surgical resection, chemotherapy and external beam irradiation have done little to alter the progression of this deadly disease. The lack of efficacy demonstrated by conventional therapies has prompted a search for other potentially beneficial therapies. The most promising approach for the treatment of brain tumors is the development of immunotherapy with different biological response modifiers (BRMs) such as IL-2, IFN-γ etc.
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Serampore, Hooghly

November 15th & 16th 2002
Azadirachta indica (neem) Leaf Water Extract Induced Activation of Immune System can Inhibit the Growth of Ehrlich Ascites Carcinoma and B16 Melanoma in Murine System

Rathindranath Baral and Utpala Chattopadhyay

Department of Immunoregulation and Immunodiagnostics, Chittaranjan National Cancer Institute,
37, S. P. Mookherjee Road, Kolkata-700 026

The present study was designed to ascertain the immunological basis of Azadirachta indica (neem) leaf water extract (AILWE) mediated protection of mice from the growth of Ehrlich ascites carcinoma (EAC) and B16 melanoma (B16 mel). Using these tumor models, conditional tumor growth inhibition was observed, when mice (swiss and C57 BL/6) was injected with AILWE (1 unit/mice/week) either before or after challenge of 1x10^6 tumor cells. Injection of AILWE after tumor challenge demonstrated no significant tumor growth inhibition in the AILWE treated group in comparison to the untreated control. On the otherhand, tumor challenge followed by 4 weekly injections with AILWE, causes significant slower tumor growth (both EAC and B16 mel) and increased survivality of mice having AILWE prophylaxis. Additionally, no cytotoxic effect of AILWE towards these two types of tumor cells was observed in vitro as revealed, by MTT assay. This conditional tumor growth retardation may be regulated by AILWE mediated immune activation, observed in mice treated with AILWE before tumor challenge.

Morphological, histological and cytometric evidences indicate the partial increase in hematopoiesis as well as increase in lymphocytic storage after AILWE treatment. These lymphocytes proliferate actively in in vitro against ConA and LPS, in comparison to those lymphocytes obtained from untreated mice. Moreover, ConA/LPS induced lymphocytic proliferation is increased significantly after combination of AILWE with mitogens. However, AILWE alone has no proliferative effect, suggesting co-stimulatory role of AILWE in mitogenic proliferation. An in vivo tumor neutralization assay (modified Winn Assay) shows slower tumor growth in mice received B16 mel tumors along with spleen cells from AILWE immune mice. Survivality of this group of mice was observed to be significantly greater than those obtained from mice having injection with nonimmune spleen cells + B16mel tumors. Supportive data was obtained from in vitro study. These evidences suggest that AILWE induced immune activation may offer better protection of mice from tumor growth, which was absent in those group of mice received AILWE treatment after tumor challenge.

The Prevalence of Resistance to Third Generation Cephalosporins Among Clinical Strains in Kolkata

S Arora and M Bal

Section Microbiology, Dept. of Physiology
Univ. Col of Sc & Tech, Calcutta University, Kolkata-700 009

Extended-spectrum β-lactamase (ESBLs) are a major cause of antibiotic resistance. These enzymes inactivate extended-spectrum β-lactam antibiotics, such as older penicillins, second and third generation cephalosporins like cefotaxime, cefazidime and related β-lactams, rendering these drugs ineffective against bacterial infection. ESBLs are found in hospitalized patients worldwide. The prevalence of ESBLs among
clinical isolates varies between countries and institutions, ranging from 0% to 40%. This resistance is usually due to plasmid acquisition. Infections caused by ESBLs are a growing clinical problem. However, there is wide variation in the level of resistance to third generation beta-lactams (cephalosporins) conferred by these enzymes.

We studied antibiotic susceptibility pattern of fifty-one clinical isolates for various beta-lactam antibiotics in Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus and Salmonella typhi collected from several hospitals and nursing homes of Kolkata. Of these the highest resistance was found in cefpodoxime 74.5% and lowest in ceftriazone 33.3%. Eighty three percent (83%) of E.Coli, 50% of S. aureus, 85.7% of K. pneumoniae and 100% of S. typhi were resistant to cefpodoxime. Whereas 66% E.Coli and 42% K. pneumoniae were resistant to ceftazidime. Using the NCCLS criteria we found that 29.4% of the organisms were ESBL producers. This incidence of resistance to extended-spectrum beta-lactam antibiotics is quite alarming.

Molecular Cloning of Snake Venom Toxins for DNA Immunization of Mice by Gene Gun

K. Bharati, R. A. Harrison and R. D. G. Theakston
Division of Molecular Biology and Immunology
Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, U.K.

Snakebite is a serious medical problem, the only conventional treatment for which is the administration of antivenom, which is produced by hyperimmurizaition of horses with either a single venom (monovalent antivenom) or a range of venoms (polyvalent antivenom). Antivenoms thus produced contain many redundant antibodies, raised against the non-toxic components of the venoms. It follows therefore that these conventional antivenoms lose their specificity due to a dilution effect. The present study describes a new toxin-targeted approach involving the cloning of individual snake venom toxins, the nucleotide sequences of which were incorporated into DNA constructs in mammalian expression plasmids for Gene Gun immunization of mice to assess their toxin-specific antibody inductive capacity.

Microbiological Quality of Common Thirst Quenchers Available in the Market of Kolkata

Dipannita Dutta, and Manjusri Bal
Microbiological Laboratory, Dept of Physiology, Calcutta University

Summer is that time of the year when we like to take various kinds of drinks to quench our thirst. Freshly squeezed orange juice, sparkling colas, green coconut water and packaged drinking water - they all quench our thirst. The vast majority of these juices are not only healthy but safe. Very rarely, however, these juices can turn dangerous. This project aimed in analyzing the microbial quality of different juices and drinks available in the market of Kolkata as thirst quenchers. A total of 18 types of samples were taken which were categorized under 6 groups. They were examined for the overall microbial quality in terms of bacterial, mold, coliform & E.coli levels. Standard procedure & media were used for isolation & identification studies. The shocking truth about the safety of consuming these juices is that the roadside juices, two of the packaged drinking waters & satchet drinks do need a serious concern of proper sanitation. The fruit juice concentrates
Among antiseizure drugs, sodium valproate (96%) was most commonly prescribed, whereas among antidepressants, venlafaxine (45.4%) was the most commonly prescribed drug.

Conclusion: This preliminary study may help us to promote rationale use of drugs which could be facilitated by periodic feedback to the prescribers.


*A Arora (Ray) Suranjana and Bai Manjusri
SECTION MICROBIOLOGY, DEPARTMENT OF PHYSIOLOGY; UNIVERSITY COLLEGE OF SCIENCE AND TECHNOLOGY; CALCUTTA UNIVERSITY; KOLKATA – 700009
*PERMANENT ADDRESS : BLDEA'S SHRI B.M. PATIL MED COL. BIJAPUR, KARNATAKA.

Detection of clinical strains with Extended-spectrum beta lactamases (ESBL) related resistance phenotypes is becoming important in clinical microbiology. One of the reliable test is Double Disk synergy test, where a beta-lactam disk and that of a beta-lactam/beta-lactamase inhibitor, are placed on a lawn of the test organism on an agar plate. If ESBL is present in the organism, the zone of inhibition around the beta-lactam/beta-lactamase inhibitor should be enhanced than that of the beta lactam disk, giving rise to an elliptical or key hole zone in between the disks. In our study we determined the in vitro activity of 12 antimicrobial agents, which included third generation cephalosporins, by disk diffusion testing and minimum inhibitory concentration (MIC) method on fifty-one hospital isolates. We identified the ESBL producers using NCCLS criteria and than confirmed it by double-disk synergy test (DDST) using discs of amoxicillin, cefotaxime, ceftriazone, cefpodoxime by themselves and combined with beta lactamase inhibitors like clavulanic acid. The disc were placed 15 mm apart on a lawn of bacteria and incubated for 18 hrs. at 35°C. The enhancement of zone of inhibition between the beta-lactam disk and that containing the beta-lactamase inhibitor was noted. Of the fifty-one clinical strains, fifteen were found to have zone diameter in DDST greater than 5 mm, phenotypically confirming ESBLs production.

RES-01 EVALUATION OF PULMONARY FUNCTION PARAMETERS IN WORKERS EXPOSED TO YARN FIBERS IN A SPINNING MILL IN LUDHIANA.

DEPARTMENT OF PHYSIOLOGY, CHRISTIAN MEDICAL COLLEGE, LUDHIANA 141008.

Adverse effects of "fly" generated during processing of yarn fibers include cough, bronchitis, byssinosis & bronchial asthma and is typically evident on the first day back to work after an absence of 48 hours or more. The objectives of the study were to assess adverse health effects of organic dusts generated during the processing of cotton fibers on textile mill workers and to compare with normal subjects who are not exposed to any pollutants. In 30 workers who were exposed to fly during the processing of cotton in a local cotton-spinning mill, a careful history was taken and a physical examination was done. Subsequently the subjects underwent spirometric remeasurements at the beginning and at the end of the first shift after a break of 48 hours and on the fourth day of the workweek. Thirty controls (age and sex matched) working in areas where there was no exposure to organic dust or other pollutants were also studied. Parameters such as FVC, PEFR, FEV1 and MVV were compared between the two groups.

RES-02 Study of Pulmonary Functions of School Going Children in Relation to Age, Height & Weight

Shraddha Singh, Deepak Saxena, S. Tewari, U. S. Pandey, Suryakant
DEPT. OF PHYSIOLOGY, CSMMU, LKO.

Pulmonary function tests are an essential part of the investigation of many respiratory diseases. The present study was planned to determine the pulmonary functions in healthy school going children of Lucknow.
Malaria has been one of the most significant idler across the globe. Chloroquine since it has proven the status of the drug. Present paper illustrates the drug chloroquine and its future. When antibiotics first came into use, microbial resistance was not a problem, but now it is widespread. For determining the resistance pattern in the pathogens around Kolkata, we collected bacterial samples from various hospitals in Kolkata over a period of four months. We found that 72% of the strains collected were ESBL producers. This is the first documented evidence of beta-lactamase producers in Kolkata. 22. When antibiotics first came into use, microbial resistance was not a problem, but now it is widespread. For determining the resistance pattern in the pathogens around Kolkata.
XVth Annual Conference
of
The Physiological Society of India
Organized by
Department of Physiology
AL-AMEEN MEDICAL COLLEGE
BIJAPUR (Karnataka)
December 4th, 5th & 6th 2003
(CME & National Conference)
H. pylori infection: relation with cardiovascular risk factors.

Vinutha Shankar M.S., Lakshmana Kumar, Y.C. Prathima K. M.,
Kutti M., Nachal A., Prasad, Sudheendra, Srikantha S.H.
Department of Physiology, Sri Devraj Urs Medical College, Kolar, Karnataka

H. pylori infection has been implicated in the etiology of peptic ulcer disease and gastric cancer. Recently interest has been developed in the association of H. pylori with cardiovascular diseases like hypertension coronary artery disease and myocardial infarction. Not many Indian studies are available regarding the same. It has been postulated that chronic H. pylori infection is accompanied by persistent inflammatory response and this may contribute to the risk of coronary heart disease by increasing the concentration of inflammatory markers like C-reactive protein, fibrinogen. Evidence is accumulating from both clinical and experimental findings that markers of inflammation correlate with coronary risk. This study aims to investigate whether cardiovascular risk factors like plasma fibrinogen and CRP levels are elevated in chronic H. pylori infections. Lipid profile and total leukocyte count were also done. Patients attending Gastroenterology OPD, RLJHC Kolar were included in the study. Presence of chronic H. pylori infections was detected by endoscopy, biopsy for rapid urease test and confirmed by histopathological examination. CRP levels were estimated by Latex agglutination test and Fibrinogen level estimated by turbidimetry. There was a significant increase in fibrinogen levels in chronic H. pylori infections. Results will be discussed during presentation.

Occurrence of AmpC β-lactamase among E. coli, K.pneumonia, K.aeruginosa, P.aeruginosa and P.vulgaris in Kolkata.

*Rorai (Ray) Surangwita & Bal M

Department of Physiology, Microbiology Lab, University of Calcutta, 92, A.P.C. Road.
Kolkata.

β-lactamases produced by bacteria are now having new substrate profile and reduced susceptibility to β-lactamases inhibitors. These new β-lactamases are an evolutionary upgrade which gives the pathogen a greater potential to resist β-lactam antibiotics and present formidable therapeutic, infection control and diagnostic challenges. AmpC β-lactamases are a new type of cephalosporinases that are poorly inhibited by clavulanic acid but are inhibited by sulbactam. We tested a total of 210 consecutive, non-repeat isolates of E. coli (114), K.pneumonia (62), K.aeruginosa (6), P.aeruginosa (15) and P.vulgaris (13) for ESBL production. Using double disc synergy test with and without clavulanic acid (CA) and sulbactam, we found that 43 were ESBL producers, 16 were AmpC producers and 4 were inducible AmpC β-lactamases producers. Three-dimensional extract method (a phenotypic test) was used to confirm AmpC β-lactamases production. This AmpC β-lactamases represents a new therapeutic threat since they confer resistance to 7-α-methoxy-cephalosporins and are not affected by CA. This type of resistance has been found around the world but have not yet been studied and reported from Kolkata. We present the first report of AmpC β-lactamases from Kolkata.
Third Congress of Federation of Indian Physiological Societies (FIPS)

24-26 November, 2000
Calcutta

Organised by:
THE PHYSIOLOGICAL SOCIETY OF INDIA
Department of Physiology
UNIVERSITY OF CALCUTTA
92, Acharya Prafulla Chandra Road
Calcutta-700 009
NADP-GDH from Aspergillus niger: A key determinant of 2-oxoglutarate flux during acidogenesis

Shahid Noor and N.S. Punekar
Biotechnology Group, SBB, IIT-Bombay, Mumbai-400076

NADP-GDH from Aspergillus niger exhibited a sigmoid saturation with respect to its specific activities decreased sharply after 24 h. While significantly high activities were maintained throughout the course of acidogenic growth (192 h). the GS activity declined after 48 h. Therefore, the increase in MICs adding clavulanic acid for CTX tested alone, suggesting inducibility of the B-lactamas*s. Inducibility of the B-lactamas*s demonstrated by the blunting of the cephalexin disks adjacent to the cefoxitin and CA acid disks. The increase in MICs adding clavulanic acid to the cephalexin was noticed, Thus a 5% of inducible B-lactamases producers were found in clinical isolates of Kolkata. So when presence of B-lactamase enzymes is suspected or tested, use of strong AmpC inducible agents could be avoided to prevent therapeutic failure.

ARGinine INDUCED ALTERATION OF STRUCTURE AND FUNCTION OF T7 RNA POLYMERASE

P. Grihan (I), Suman Das, Mill Das
Biophysics Division, Saha Institute of Nuclear Physics, 37 Belgachia Road, Kolkata-700 037 India

We reported earlier an alteration in the tertiary structure leading to an enhancement of transcriptional activity for T7 RNA polymerase in the presence of low concentrations of antioxidant containing guaodine group. In order to understand the process further we have studied the effect of an amino acid, arginine, which contains the guaodine group, upon the structure and activity of T7 RNA polymerase. We have examined the structure by intrinsic fluorescence, near UV CD, thermal melting and limited proteolysis of the enzyme. Results from all these experiments suggest an alteration in the tertiary structure of the enzyme leading to an enhancement in the transcriptional activity as monitored from full length transcriptional assay using radiolabeled UTP. Significance of these results will be discussed with reference to switch-over from abortive to processive transcriptional activity of the enzyme in presence of the amino acid.
CHARACTERISTICS OF CLINICAL ISOLATES OF KOLKATA USING NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS (NCCLS) EXTENDED-SPECTRUM BETA-LACTAMASE DETECTION METHOD

*Arora (Ray) Suranjana* and Bal Manjusri
Section Microbiology; Department of Physiology; University College of Science and Technology; Calcutta University Kolkata-700 009.

*B.L.D.E.A's Sri.B.M.Patil Medical College & Research Centre, Bijapur, Karnataka.*

**ABSTRACT**

Extended-spectrum beta-lactamases (ESBLs) are enzymes found in bacteria that mediate resistance to extended-spectrum cephalosporins. The detection of ESBL in any hospital has a great significance from the point of view of infection control. In 1999, the National Committee for Clinical Laboratory Standards (NCCLS) published methods for screening and confirming the presence of ESBLs in clinical isolates. We tested 75 clinical isolates (25 E.coli, 25 K.pneumoniae & 25 S.aureus) from 5 different hospitals in Kolkata. Using the NCCLS screening criteria for ESBL production (resistance to at least two third generation cephalosporin and reduction in the cephalosporin MIC of more than 3 doubling dilution steps, for the agent tested alone versus its MIC when tested in combination in clavulanic acid). We identified 20 isolates (26.7%) as ESBL producers which we confirmed by using by double disc synergy test (>= 5 mm increase in zone of inhibition around the disc containing clavulonic acid). The laboratory identification of ESBL detection is still in an evolving state. The detection of ESBL producing strains is important for optimal therapy of infected patients. An ESBL producing isolates may predict therapeutic failure with extended spectrum cephalosporin drugs.

* Presenting Author.
CHROMOGENIC TESTS FOR BETA-LACTAMASE PRODUCTION

Arora (Ray) Suranjana** and Bal Manjusri*

*Dept of Physiology, Univ Col. Of Sc & Tech, Calcutta University; Kolkata- 700 009
**Dept of Physiology, Shadan Institute of Medical Science; Hyderabad-500 008

ABSTRACT

Beta-lactamases are the commonest cause of bacterial resistance to beta-lactam antibiotics. Among tests for beta-lactamase production chromogenic methods are faster and convenient. We studied 284 clinical isolates from 6 hospitals of Kolkata for production of beta-lactamases. Of these we found 85 isolates (29.9%) to be beta-lactamase producers by Nitrocephin spot test (NST) (where the presence of beta-lactamase was detected if color of Nitrocephin changed from yellow to pinkish-red) and 109 isolates (38.4%) were found to produce beta-lactamase by Microiodometric test (MIT) (decolorisation of starch-iodine complex) using benzyl-penicillin and cefotaxime as substrate. The MIT is cheaper than NST, and given care, almost as sensitive, but is more prone to false positive results.

93rd Science Congress, Hyderabad, Jan 2006