International Journal of Infectious Diseases (2008) **12**, 256—264

http://intl.elsevierhealth.com/journals/ijid

Prevalent phenotypes and antibiotic resistance in *Escherichia coli* and *Klebsiella pneumoniae* at an Indian tertiary care hospital: plasmid-mediated cefoxitin resistance

M. Shahid [a,*], A. Malik [a], M. Akram [b], L.M. Agrawal [a], A.U. Khan [b], M. Agrawal [a]

[a] *Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh 202002, Uttar Pradesh, India*
[b] *Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, Uttar Pradesh, India*

Received 19 August 2006; received in revised form 18 August 2007; accepted 30 August 2007
Corresponding Editor: J. Peter Donnelly, Nijmegen, The Netherlands

KEYWORDS
Resistance phenotypes;
Escherichia coli;
Klebsiella pneumoniae;
Spot-inoculation method;
Modified three-dimensional test;
Cefoxitin resistance

Summary
Background: The β-lactam antibiotics, in combination with aminoglycosides, are among the most widely prescribed antibiotics. However, because of extensive and unnecessary use, resistance to these drugs continues to increase. In recent years, resistance in the Indian bacterial population has increased markedly, the majority showing complex mechanisms. Due to increased transcontinental movement of the human population, it would be wise to know the prevalence and resistance complexity of these strains, well in advance, in order to formulate a policy for empirical therapy.
Methods: One hundred and eighty-one isolates of *Escherichia coli* and 61 isolates of *Klebsiella pneumoniae* obtained from 2655 non-repeat samples of pus (912) and urine (1743) were studied, and their resistance rates and patterns were noted. The isolates were analyzed for prevalent aminoglycoside and cephalosporin resistance phenotypes and for the presence of extended spectrum β-lactamase (ESBL) and AmpC enzymes by spot-inoculation and modified three-dimensional tests developed in our laboratory. Fourteen isolates of *E. coli* and six of *K. pneumoniae*, resistant to all of the antibiotics tested, were selected for plasmid screening, curing, and transconjugation experiments, and for comparative evaluation of the double disk synergy test (DDST) and modified three-dimensional test (TDT) for detection of β-lactamases.
Results: Urinary *E. coli* isolates showed maximum susceptibility to amikacin (57.1%), followed by tobramycin (38.5%) and gentamicin (31.9%). Eighteen (19.8%) isolates were susceptible to cefotaxime, whereas 11 (12.1%) were susceptible to ceftriaxone. The *K. pneumoniae* isolates from urine samples showed maximum susceptibility to tobramycin (63.6%) followed by amikacin (54.5%). Of the *K. pneumoniae* isolates, 31.8% were susceptible to cefotaxime and 13.6% were

* Corresponding author. Tel.: +91 571 2720382; fax: +91 571 2721776.
E-mail address: shahidsahar@yahoo.co.in (M. Shahid).

doi:10.1016/j.ijid.2007.08.008

susceptible to ceftriaxone. A more or less similar trend of antibiotic susceptibility was noted in *E. coli* and *K. pneumoniae* isolates from pus samples. Twenty-six (14.4%) *E. coli* and 15 (24.6%) *K. pneumoniae* isolates were found to be ESBL-producers by NCCLS-ESBL phenotypic confirmatory test. Eighteen (9.9%) *E. coli* and 19 (31.1%) *K. pneumoniae* isolates were found to be AmpC enzyme-producers by our modified TDT. The simultaneous occurrence of ESBL and AmpC enzymes was noted in 7.7% and 9.8% isolates of *E. coli* and *K. pneumoniae*, respectively.

Conclusions: The prevalence of multidrug-resistant bacterial isolates is quite high in our bacterial population. On comparative evaluation of DDST and TDT in resistant isolates, TDT was found to be the better method, detecting ESBLs in 80% of isolates compared to 15% with DDST. A 19.9-kb plasmid was consistently present in all the screened isolates of *E. coli* and *K. pneumoniae*, and was inferred to encode cefoxitin and tetracycline resistance based on curing and transconjugation experiments.

Introduction

An increase in the emergence of multidrug-resistant bacteria in recent years is worrying the world population. The presence of antibiotic resistance genes on bacterial plasmids has further helped in the transmission and spread of drug resistance among pathogenic bacteria.[1,2] To combat antibiotic resistance, a broad array of potent antibiotics is available to present-day clinicians. However, growing problems with antimicrobial drug resistance are beginning to erode our antibiotic armamentarium.[3]

The β-lactam antibiotics, in combination with aminoglycosides, are among the most widely prescribed antibiotics and are important components of empirical therapy. Because of extensive and unnecessary use in developing countries, resistance to these drugs has become a major problem especially after the introduction of newer broad-spectrum cephalosporins, β-lactamase inhibitor/β-lactam antibiotics, monobactams, and carbapenems. A major feature in the emergence of multidrug-resistant Gram-negative bacilli is the production of extended-spectrum β-lactamases (ESBLs) and enzymatic modification of aminoglycosides, which are responsible for resistance to β-lactam antibiotics and aminoglycosides, respectively. Plasmid-mediated ESBLs in a variety of Gram-negative bacterial species have been reported from various developed countries;[4] however, data on this subject and the prevalent aminoglycoside and cephalosporin-resistant phenotypes is rudimentary from Indian hospitals. Plasmid-encoded AmpC β-lactamases are produced by numerous pathogens.[5] However, the true rate of occurrence of AmpC enzymes has not yet been extensively studied, especially not in Indian isolates.[6] Hence, the present study was designed to determine the resistance rates and patterns in *Escherichia coli* and *Klebsiella pneumoniae* isolates, to infer the prevalent resistance phenotypes, to detect the ESBLs and AmpC β-lactamases by spot-inoculation and modified three-dimensional test, and to screen a few isolates for R-plasmids and plasmid-mediated antibiotic resistance.

Materials and methods

Bacterial isolates

A total of 2655 non-repeat samples of pus (n = 912) and urine (n = 1743) obtained from hospitalized patients at Jawaharlal Nehru Medical College and Hospital, India, during the period from 1 May to 31 July 2003, were subjected to routine culture and susceptibility testing. The isolates of *E. coli* (n = 181) and *K. pneumoniae* (n = 61) were included in the study, and resistance rates and patterns were noted.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed according to the method of Bauer et al.[7] on Mueller–Hinton agar (HiMedia, India) by using commercial antibiotic disks (HiMedia, India). For *E. coli* isolates, the antibiotics used (μg/disk) were: ampicillin (10), co-trimoxazole (trimethoprim–sulfamethoxazole, 1.2/23.8), tetracycline (30), amikacin (30), gentamicin (10), tobramycin (10), ciprofloxacin (5), cefotaxime (30), and ceftriaxone (30). For *K. pneumoniae*, all the above-mentioned antibiotics were used, except ampicillin. Nitrofurantoin (300 μg) and norfloxacin (10 μg) were also used for isolates from urine samples. The results were interpreted as per the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS).[8]

Determination of aminoglycoside/cephalosporin resistance phenotypes and possible resistance mechanisms

Aminoglycoside and cephalosporin resistance phenotypes and resistance mechanisms were inferred by 'interpretative reading'.[9] A range of cephalosporins and aminoglycosides (HiMedia, India) along with some other classes of antibiotics (HiMedia, India) were used for determination of resistance phenotypes and to interpret the cephalosporin and aminoglycoside resistance mechanisms.

All the isolates of *E. coli* and *K. pneumoniae* were tested to infer aminoglycoside resistance phenotypes and possible mechanisms of aminoglycoside resistance. The aminoglycoside antibiotics used (μg/disk) were: gentamicin (10), amikacin (30), tobramycin (10), kanamycin (30), neomycin (30), and netilmicin (30). Since a large panel of antibiotics is required to infer the mechanism of cephalosporin resistance, the isolates found to be ESBL-producers by NCCLS phenotypic confirmatory test and AmpC-producers by three-dimensional test (TDT), were tested to determine cephalosporin resistance phenotypes and possible mechanisms of cephalosporin resistance. The antibiotics used to detect cephalosporin resistance phenotypes and mechanisms (μg/disk) were:

ampicillin (10), amoxicillin–clavulanate (20/10), ticarcillin (75), ticarcillin–clavulanate (75/10), piperacillin (100), piperacillin–tazobactam (100/10), cephalothin (30), cefuroxime (30), ceftriaxone (30), cefepime (30), cefoxitin (30), aztreonam (30), and imipenem (10).

ESBL detection by NCCLS phenotypic method

The NCCLS-ESBL phenotypic confirmatory test with ceftazidime (CAZ)[10] was used for all the isolates by the disk diffusion method. Mueller–Hinton agar plates and disks containing 30 μg of CAZ, with and without 10 μg of clavulanic acid (CA), were used. Susceptibility test results were interpreted according to the criteria established by the NCCLS.[10] A ≥5-mm increase in the zone diameter of CAZ tested in combination with CA versus its zone when tested alone, was considered indicative of ESBL production. *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* 700603 were used as control strains.

Detection of AmpC β-lactamases

All the isolates of *E. coli* and *K. pneumoniae*, irrespective of the cefoxitin zone diameters, were screened for the presence of AmpC enzymes by spot-inoculation method as previously described.[11] The screen-positive isolates were further confirmed phenotypically for the presence of AmpC enzymes by modified TDT as previously described.[11]

A limited number of isolates that were found resistant to all of the primary screening antibiotics and not giving any zone of inhibition (*E. coli*, $n = 14$; *K. pneumoniae*, $n = 6$), were selected for detection of R-plasmids, curing experiments, transconjugation experiments, random amplification of polymorphic DNA (RAPD)-polymerase-chain reaction (PCR) typing, and for comparison of ESBL detection by double disk synergy test (DDST) and modified TDT.

Screening for R-plasmids

The selected isolates of *E. coli* and *K. pneumoniae* were screened for R-plasmids by the alkaline lysis method of Davis et al.,[12] as adopted in our previous studies.[6,13] Plasmid samples (25 μl) were electrophoresed through 0.8% agarose (type 1, Sigma) containing ethidium bromide (0.5 μg/ml) in TBE buffer at 150 V, 60 mA for 3 h by the method of Portnoy et al.[14] A 1-kb ladder (Promega Corp., Madison, WI, USA) was used as a reference molecular weight marker. Plasmid bands were analyzed by gel documentation system (Vilber Lourmat, France), and their respective molecular weights were calculated with the help of PhotoCaptMW Software provided with the gel documentation system.

Curing experiments

For curing experiments, the method described by Guerry and Colwell[15] was used with slight modifications as described elsewhere.[13,16] Ethidium bromide (Sibisco Research Lab. Ltd, India) was used as the curing agent. The minimal inhibitory concentration (MIC) of ethidium bromide was determined for the bacterial isolates in trypticase soy broth (TSB), and the highest concentration permitting growth was used for plasmid curing. Subcultures were initially done on trypticase soy agar and the colonies were tested for antibiotic susceptibility. Concomitant loss of the antibiotic resistance was compared with the loss of plasmid content.

Transconjugation experiments

Transconjugation experiments were done following the method of Abigail et al.[17] Mating was performed with *E. coli* K12J62-2 ($F^-rif^+lac^-$) as recipient strain. Transconjugants were selected on MacConkey agar supplemented with rifampin (2.5 mg/ml) and cefoxitin (20 μg/ml).

RAPD-PCR typing

RAPD typing of the R-plasmid-harboring isolates was done according to the method described by Vogel et al.[18] to determine any predominant clone. Template DNA was prepared from bacteria grown overnight at 37 °C on nutrient agar plates. Crude DNA extracts were obtained by suspending a colony in 50 μl of distilled water and boiling at 95 °C for 5 min. Each RAPD-PCR reaction consisted of Ready-To-Go PCR bead (Amersham Biosciences, Amersham, UK), 25 pmol of primer ERIC-2b, and 2 μl of DNA template in a final reaction volume of 25 μl. The cycling conditions were as follows: one cycle at 94 °C for 2 min; 35 cycles at 94 °C for 1 min, 25 °C for 1 min, and 72 °C for 4 min; and a final cycle at 94 °C for 1 min, 25 °C for 1 min, and 72 °C for 8 min. RAPD products were separated on a 1.5% agarose gel containing ethidium bromide (0.5 mg/l) with TAE running buffer. Molecular size marker ladders (100-bp; Bioline, London, UK) were included on all gels. Isolates of the same species were typed in the same batch and products run on the same gel. Banding patterns were evaluated using PhotoCaptMW software (Vilber Lourmat, USA). The whole RAPD experimentation was performed in duplicate to look for any major differences in the banding patterns of individual isolates.

Comparative analysis of ESBL detection by double disk synergy test (DDST) and three-dimensional test (TDT)

The method of Jarlier et al.,[19] with some modifications, was used for the detection of ESBL by DDST. Ceftazidime (30 μg) and amoxicillin–clavulanate (20/10) were used. Taking into consideration the controversies on optimal disk placement, various modifications were tried, putting the CAZ and amoxicillin–clavulanate disks at 5, 10, 15, 20, and 30 mm apart. For determination of ESBL by TDT, the isolates were first screened by spot-inoculation method,[11] and then further confirmed by the modified TDT as described previously.[11]

Results

Of the 2655 samples that were cultured, 528 (19.9%) yielded bacterial growth, of which 139 (26.3%) bacterial isolates were obtained from urine samples and 389 (73.7%) from pus samples. The comparative results of the bacterial isolates obtained from urine and pus samples are shown in Figure 1. Only the *E. coli* ($n = 181$) and *K. pneumoniae* ($n = 61$) isolates were included for further studies.

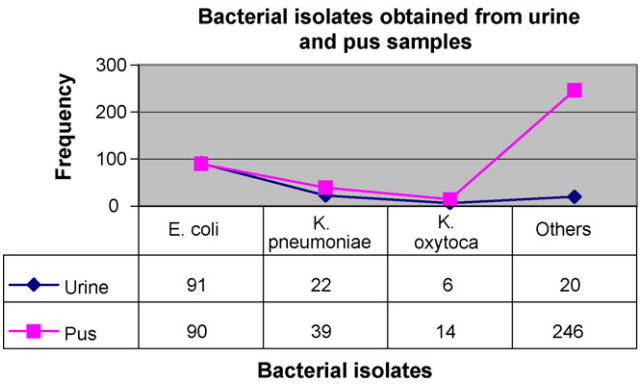

Figure 1 Bacterial isolates obtained from urine and pus samples.

Antibiotic susceptibility

The results of the antibiotic susceptibility testing are shown in Table 1. The isolates of *E. coli* obtained from urine samples showed maximum susceptibility to amikacin (57.1%) followed by tobramycin (38.5%) and gentamicin (31.9%). Eighteen (19.8%) isolates were susceptible to cefotaxime, whereas 11 (12.1%) were susceptible to ceftriaxone. The *K. pneumoniae* isolates from urine samples showed maximum susceptibility to tobramycin (63.6%) followed by amikacin (54.5%). Of the *K. pneumoniae* isolates, 31.8% were susceptible to cefotaxime and 13.6% were susceptible to ceftriaxone. A more or less similar trend of antibiotic susceptibility was noticed in *E. coli* and *K. pneumoniae* isolates from pus samples (Table 1).

Aminoglycoside resistance phenotypes and possible resistance mechanisms

The aminoglycoside resistance phenotypes of *E. coli* obtained from urine and pus samples are shown in Table 2. Of the *E. coli* isolates from urine samples, the most common resistance phenotype was GnNtTbAkKnNe, noted in 29 (31.9%) isolates followed by Gn and GnNtTbKn(r)Ne, which were noted in 14 (15.4%) isolates of *E. coli* each.

Inferring the resistance mechanism, the most common resistance mechanism noted in 31.9% isolates was due to impermeability. The most common aminoglycoside-modifying enzymes inferred by interpretative reading were AAC(3)-I and AAC(3)-IV, followed by AAC(6′). However, 13 (14.3%) isolates could not be characterized on the basis of interpretative reading (Table 2). Among the *E. coli* isolates from pus samples, again GnNtTbAkKnNe was found to be the most common resistance phenotype in 27 (30%) isolates. The most common aminoglycoside-modifying enzyme inferred was AAC(3)-II in 10 (11.1%) isolates followed by AAC(3)-I and AAC(6′) in nine (10%) and eight (8.9%) isolates, respectively. Sixteen (17.8%) isolates could not be characterized by interpretative reading (Table 2).

The aminoglycoside resistance phenotypes of *K. pneumoniae* obtained from urine and pus samples are given in Table 3. Scattered frequencies of resistance phenotypes were noted in isolates from urine samples and the most common aminoglycoside-modifying enzymes inferred were AAC(6′) and ANT(2′) noted in 13.6% isolates each. Among the isolates from pus samples, the most common resistance phenotype was GnNtTbAkKnNe, noted in 33.3% isolates, and impermeability was inferred as the possible resistance mechanism. The most common aminoglycoside-modifying enzymes inferred by interpretative reading were APH(3′) and AAC(3)-II. However, 12.8% isolates could not be characterized on the basis of interpretative reading (Table 3).

Cephalosporin resistance phenotypes and possible resistance mechanisms

The cephalosporin resistance phenotypes of *E. coli* and *K. pneumoniae* and their possible resistance mechanisms are shown in Table 4. Fourteen (7.7%) *E. coli* isolates and six (9.8%) *K. pneumoniae* could not be characterized by interpretative reading because of complex resistance patterns (i.e., simultaneous occurrence of ESBLs and AmpC enzymes (Table 4)).

Occurrence of ESBL-producers

On the basis of NCCLS-ESBL phenotypic confirmatory test, 26 (14.4%) *E. coli* isolates and 15 (24.6%) *K. pneumoniae* were

Table 1 Percent antibiotic susceptibility of *Escherichia coli* and *Klebsiella pneumoniae* isolates from urine and pus samples against the first-line antibiotics tested

Antibiotics	Urine		Pus	
	E. coli (*n* = 91)	*K. pneumoniae* (*n* = 22)	*E. coli* (*n* = 90)	*K. pneumoniae* (*n* = 39)
Ampicillin	3.3 (3)	NT	2.2 (2)	NT
Co-trimoxazole	11.0 (10)	4.5 (1)	2.2 (2)	0 (0)
Tetracycline	8.8 (8)	9.1 (2)	4.4 (4)	12.8 (5)
Ciprofloxacin	23.1 (21)	31.8 (7)	22.2 (20)	28.2 (11)
Gentamicin	31.9 (29)	45.5 (10)	24.4 (22)	30.8 (12)
Amikacin	57.1 (52)	54.5 (12)	46.7 (42)	35.9 (14)
Tobramycin	38.5 (35)	63.6 (14)	31.1 (28)	46.2 (18)
Cefotaxime	19.8 (18)	31.8 (7)	15.6 (14)	7.7 (3)
Ceftriaxone	12.1 (11)	13.6 (3)	5.6 (5)	7.7 (3)
Nitrofurantoin	11.0 (10)	9.1 (2)	NT	NT
Norfloxacin	23.1 (21)	31.8 (7)	NT	NT

Figures are % (number of isolates). The isolates were resistant to multiple antibiotics. NT = not tested.

Table 2 Aminoglycoside resistance phenotypes and mechanisms of resistance in *Escherichia coli* isolates

Resistance phenotypes	Interpretation	% Isolation from urine (*n*)	% Isolation from pus (*n*)
GnNtTbAkKnNe	Impermeability	31.9 (29)	30 (27)
Susceptible to all	Classical	15.4 (14)	6.7 (6)
Gn	AAC(3)-I	15.4 (14)	10 (9)
NtTbAkKnNe	AAC(6′)	6.6 (6)	8.9 (8)
KnNe	APH(3′)	1.1 (1)	2.2 (2)
GnNtTbKn(r)Ne	AAC(3)-IV	15.4 (14)	5.6 (5)
GnNtTbKn(r)	AAC(3)-II	—	11.1 (10)
GnTbKn	ANT(2′)	—	7.8 (7)
GnNtAkKnNe	Unclassified 1	4.4 (4)	—
NtAkKnNe	Unclassified 2	1.1 (1)	4.4 (4)
TbKnNe	Unclassified 3	8.8 (8)	—
GnAk	Unclassified 4	—	8.9 (8)
Tb	Unclassified 5	—	4.4 (4)
Total	—	100 (91)	100 (90)

Antibiotics: Gn = gentamicin, Nt = netilmicin, Tb = tobramycin, Ak = amikacin, Kn = kanamycin, Ne = neomycin. r = reduced zones but likely to remain susceptible at British Society for Antimicrobial Chemotherapy (BSAC) breakpoints.

Table 3 Aminoglycoside resistance phenotypes and mechanisms of resistance in *Klebsiella pneumoniae* isolates

Resistance phenotypes	Interpretation	% Isolation from urine (*n*)	% Isolation from pus (*n*)
GnNtTbAkKnNe	Impermeability	18.2 (4)	33.3 (13)
Susceptible to all	Classical	22.7 (5)	10.3 (4)
Gn	AAC(3)-I	4.5 (1)	2.6 (1)
NtTbAkKnNe	AAC(6′)	13.6 (3)	7.7 (3)
KnNe	APH(3′)	9.1 (2)	12.8 (5)
GnNtTbKn(r)	AAC(3)-II	4.5 (1)	12.8 (5)
GnKn	ANT(2′)	13.6 (3)	7.7 (3)
GnNtAkKn	Unclassified 1	13.6 (3)	—
GnAkKn	Unclassified 2	—	12.8 (5)
Total	—	100 (22)	100 (39)

Antibiotics: Gn = gentamicin, Nt = netilmicin, Tb = tobramycin, Ak = amikacin, Kn = kanamycin, Ne = neomycin. r = reduced zones but likely to remain susceptible at British Society for Antimicrobial Chemotherapy (BSAC) breakpoints.

found to be ESBL-producers (Table 4). Of the 26 isolates of *E. coli*, nine (34.6%) were obtained from urine samples and 17 (65.4%) were from pus samples. Among the *K. pneumoniae* isolates, four (26.7%) were from urine samples and 11 (73.3%) were from pus samples.

Occurrence of AmpC enzymes

As there are no NCCLS phenotypic confirmatory criteria for detection of AmpC β-lactamases, all the isolates of *E. coli* and *K. pneumoniae* were tested by TDT for the presence of AmpC β-lactamases. Eighteen (9.9%) isolates of *E. coli* and 19 (31.1%) isolates of *K. pneumoniae* were found to be AmpC enzyme-producers by TDT (Table 4 and Figure 2). Most of the isolates of *E. coli* and *K. pneumoniae* identified to be AmpC producers by TDT either did not have or had a very small zone of inhibition ranging between 8 and 10 mm in diameter. Comparing the cefoxitin zone diameters of the isolates with TDT-positivity, all but one isolate had zone diameter lower than the cut-off criterion, i.e., 18 mm zone diameter. One isolate of *E. coli* had a zone diameter of 22 mm.

Simultaneous occurrence of ESBLs and AmpC enzymes

Simultaneous occurrence of ESBLs and AmpC enzymes were noted in 14 isolates of *E. coli* (7.7%) and six isolates of *K. pneumoniae* (9.8%) (Table 4). All the isolates were obtained from pus samples. These 20 isolates were further selected for plasmid screening, curing, and transconjugation experiments, RAPD-PCR typing, and comparative analysis of ESBL detection by DDST and TDT.

Plasmid analysis, plasmid curing, and transconjugation experiments

All the isolates of *E. coli* and *K. pneumoniae* selected for plasmid analysis were found to harbor a single plasmid of 19.9 kb (Figure 3). Curing and transconjugation experiments were attempted in these isolates to determine the change in plasmid content associated with the antibiotic resistance pattern. The MICs of the ethidium bromide ranged between 400 and 600 μg/ml for these isolates. The loss of antibiotic

Table 4 Occurrence of β-lactamase and cephalosporin resistance phenotypes and mechanisms of resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates

β-Lactamases	% Positivity (*n*)			Resistance phenotypes (*n*)		Resistance mechanism[a]
	Total (242)	*E. coli* (181)	*K. pneumoniae* (61)	*E. coli*	*K. pneumoniae*	
ESBL alone	8.7 (21)	6.6 (12)	14.8 (9)	AmAx/ClTiTi/ClPiPi/ TzCfCzCtCpAz (6)	—	ESBL-broad
				AmTiPiPi/TzCfCzCtCpAz (1)	—	ESBL-broad
				AmTiPiPi/TzCfCzCt(r) Cp(r)Az(r) (3)	—	ESBL-ceftazidimase
				AmTiPiCfCzCt(r) Cp(r)Az(r) (2)	—	ESBL-ceftazidimase
				—	AmAx/ClTiTi/ClPiPi/ TzCfCzCtCpAz (5)	ESBL-broad
				—	AmTiPiPi/TzCfCzCtCpAz (2)	ESBL-broad
				—	AmTiPiPiTzCfCzCt(r) Cp(r)Az(r) (2)	ESBL-ceftazidimase
AmpC alone	7.0 (17)	2.2 (4)	21.3 (13)	AmAx/ClTiTi/ClPiPi/ TzCfCxCzCtAz (4)	—	AmpC high
				—	AmAx/ClTiTi/ClPiPi/ TzCfCxCzCtAz (13)	AmpC acquired
AmpC + ESBL	8.3 (20)	7.7 (14)	9.8 (6)	AmAx/ClTiTi/ClPiPi/ TzCfCxCzCtCpAz (14)	AmAx/ClTiTi/ClPiPi/ TzCfCxCzCtCpAz (6)	Unclassified by interpretative reading

Antibiotics: Am = ampicillin, Ax/Cl = amoxicillin—clavulanate, Ti = ticarcillin, Ti/Cl = ticarcillin—clavulanate, Pi = piperacillin, Pi/Tz = piperacillin—tazobactam, Cf = cephalothin, Cx = cefoxitin, Cz = ceftazidime, Ct = ceftriaxone, Cp = cefepime, Az = aztreonam. r = reduced zones but likely to remain susceptible at British Society for Antimicrobial Chemotherapy (BSAC) breakpoints.

[a] Inferred according to interpretative reading (see Ref. 15). Figures in parentheses indicate number of isolates.

resistance was concomitant with the loss of plasmid content. It was noted that all the *K. pneumoniae* isolates that lost plasmids became susceptible to cefoxitin and tetracycline, while remaining resistant to cephalosporins (not giving any zone of inhibition to cephalosporins after curing experiments) and other antibiotics. Among the *E. coli* isolates, 10 (71.4%) became susceptible to cefoxitin and tetracycline while remaining resistant to cephalosporins and other antibiotics, and did not gave any zone of inhibition to these antibiotics. However, four (28.6%) isolates revealed cephalosporin zone diameters ranging between 10 and 16 mm, in addition to susceptibility to cefoxitin and tetracycline. After

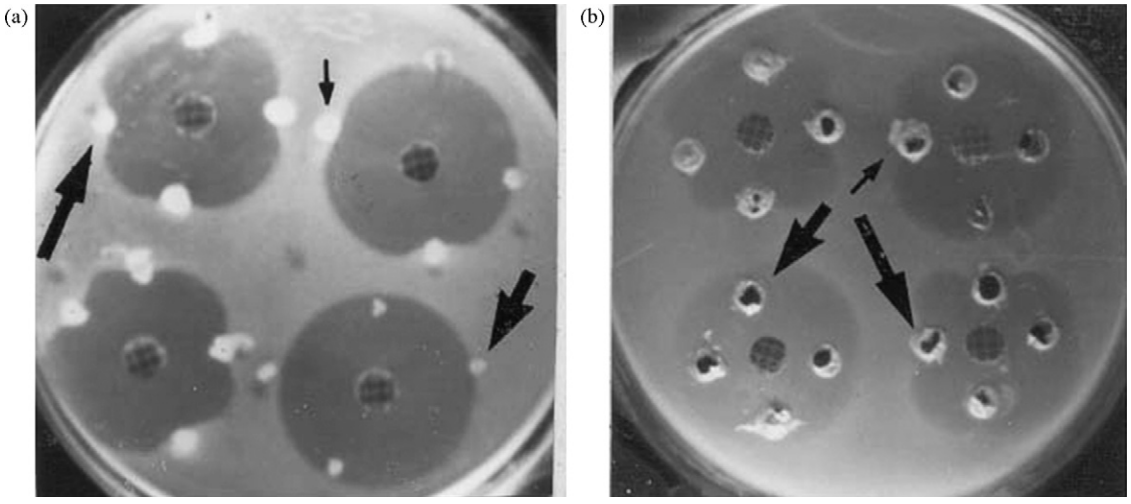

Figure 2 Three-dimensional test (TDT) for detection of β-lactamases (ESBLs and AmpC type). Ceftazidime disks (30 μg) were used for detection of ESBLs and cefoxitin (30 μg) was used for AmpC detection. (a) Spot-inoculation method. The isolates showing either flattening (small arrow) or clear distortion (large arrow) were taken as ESBL/AmpC producers. The isolates with no distortion (medium arrow) were taken as ESBL/AmpC non-producers. (b) Modified three-dimensional extract test. Flattening (small arrow) and clear distortion (large arrow) show ESBL/AmpC producers and no distortion (medium arrow) shows non-producers.

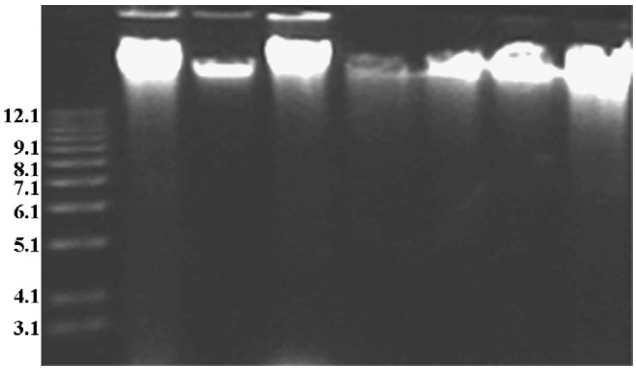

Figure 3 Agarose gel (0.8%) showing 19.9-kb plasmids. Molecular weight markers along with their sizes (in kb pairs) are shown on the extreme left.

repeated attempts, all the isolates subjected to transconjugation experiments were able to transfer resistance to cefoxitin and tetracycline. Plasmid analysis of the transconjugants revealed the presence of a similar 19.9-kb plasmid.

RAPD analysis

The isolates could not be grouped into clusters because of unique banding patterns (see Figure 4). No gross banding difference was noticed between two RAPD-PCR experiments.

Comparison between DDST and TDT

ESBLs were detected in three (15%) isolates by DDST in the experiments where ceftazidime and amoxicillin–clavulanate disks were placed 5 mm apart. The rest of the experiments with disks placement at 10, 15, 20, and 30 mm did not demonstrate the presence of ESBLs. On the other hand, ESBLs were detected in 16 (80%) isolates by TDT.

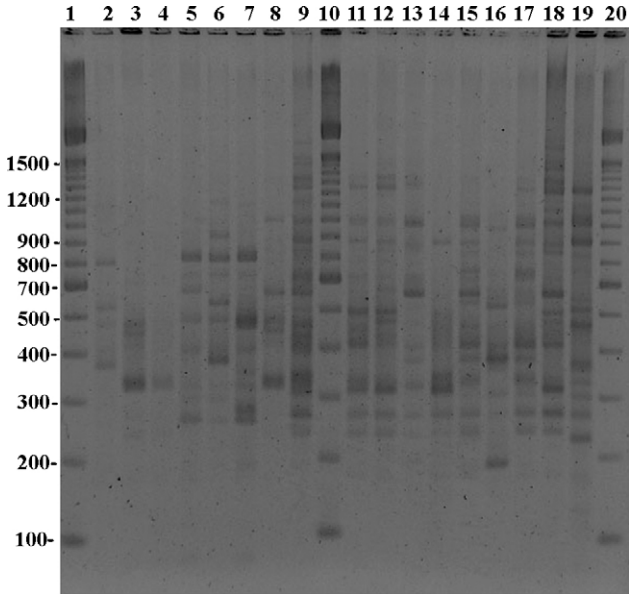

Figure 4 Agarose gel (1.5%) showing the RAPD profiles of the *Escherichia coli* isolates. Lanes 1, 10, and 20 have molecular weight markers. Lanes 11 and 12 show the RAPD pattern of a single *E. coli* isolate, in duplicate experiments demonstrating no significant difference.

Discussion

The increase in multidrug-resistant strains of *E. coli* and *K. pneumoniae* producing the ESBLs and AmpC enzymes has limited therapeutic options.[6,20] Therefore, early identification of infections due to these organisms is necessary as prompt institution of appropriate treatment might reduce the mortality in hospitalized patients.[21,22] In our study, markedly high resistance to cephalosporins and aminoglycosides was noticed in clinical isolates of *E. coli* and *K. pneumoniae*. Compared with earlier reports that have examined the susceptibility to various antibiotics of *E. coli* and *K. pneumoniae*,[23] there is a clear tendency towards decreased susceptibility for all groups of antibiotics.

Impermeability was inferred to be the most common aminoglycoside resistance mechanism in our *E. coli* and *K. pneumoniae* isolates. AAC(3)-I was the most common aminoglycoside-modifying enzyme inferred in 23 (12.7%) isolates of *E. coli*. However scattered frequencies of resistance phenotypes were inferred in *K. pneumoniae* isolates, and APH(3′) was inferred to be the most common aminoglycoside-modifying enzyme. Regarding the cephalosporin/cephamycin resistance mechanisms, production of ESBL was noticed in 16.9% of isolates, whereas the production of AmpC enzyme was noted in 15.3% of isolates. It must be emphasized here that simultaneous occurrence of AmpC and ESBL was noticed in 8.3% of isolates, in which the mechanism could not be inferred by interpretative reading. This is the first report from India regarding the aminoglycoside/cephalosporin resistance mechanisms, and our results could not be compared to others due to the paucity of Indian studies on this subject.

ESBLs are widespread all over the world, but the prevalence and phenotypic characteristics among clinical isolates may vary between geographical areas.[24,25] In the present study, we noticed the occurrence of ESBLs in 14.3% *E. coli* isolates and 24.5% of *K. pneumoniae* by NCCLS phenotypic test, and this is in concordance with earlier published reports.[23,24] AmpC enzymes were noticed in 9.9% *E. coli* and 31.1% *K. pneumoniae* isolates in our study. The prevalence of AmpC β-lactamases in this study is significantly high as compared to that found in earlier reported studies from other countries,[26,27] however it is in agreement with the prevalence found in a few reports published from India.[28] It is interesting to note that the simultaneous occurrence of ESBLs and AmpC enzymes was found in a fair percentage of isolates (7.7% and 9.8% isolates of *E. coli* and *K. pneumoniae*, respectively). This is among the first reports demonstrating a high frequency of simultaneous occurrence of ESBL and AmpC enzymes in *E. coli* and *K. pneumoniae* isolates, particularly one of the first from India.

The acquisition of AmpC-type genes by plasmids in *E. coli* and *K. pneumoniae* has been known of since the 1980s.[4] However studies regarding plasmid-mediated AmpC β-lactamases are rudimentary in our country. In this study, we found that the cause of cefoxitin resistance in *E. coli* and *K. pneumoniae* isolates from our hospital is the acquisition of a similar self-transmissible plasmid of 19.9 kb. Observing the similar plasmids in different isolates, we attempted to look

for any predominant clone that exists in our bacterial population by RAPD-PCR typing. No predominant clone was identified, indicating horizontal transfer of the 19.9 kb plasmids between unrelated strains as well as widespread dissemination between the genera (Escherichia and Klebsiella). Gazouli et al.[29] have also reported that *E. coli* and *K. pneumoniae* isolates from a Greek hospital acquired cefoxitin resistance due to the acquisition of a plasmid of 8.3 kb.

In summary, the prevalence of multidrug-resistant bacterial isolates is quite high in our locality. The prevalence of ESBLs by phenotypic NCCLS criteria was found to be 14.3% in *E. coli* isolates and 24.5% in *K. pneumoniae* isolates, and the prevalence of AmpC enzymes by phenotypic detection (TDT) was found to be 9.9% in *E. coli* isolates and 31.1% in *K. pneumoniae* isolates. Combinations of aminoglycoside-modifying enzymes were found responsible for aminoglycoside resistance, whereas the most common inferred cephalosporin resistance phenotypes were 'ESBL-broad'. The modified TDT was found to be the better method for ESBL detection as compared to the DDST, especially in isolates not giving any zone of inhibition to cephalosporins where we could not judge the optimal placement of cephalosporin and amoxicillin–clavulanate disks. A 19.9-kb plasmid was consistently present in all the isolates of *E. coli* and *K. pneumoniae* that were inferred to encode cefoxitin resistance based on curing and transconjugation experiments. RAPD analysis shows that no predominant clone exists; the bacterial population is rather diverse and horizontal transfer of the plasmids has occurred between unrelated strains.

Acknowledgements

The authors wish to thank the Department of Science and Technology, Ministry of Science and Technology, Government of India (Grant No. SR/PF/171-B/2004-2005), the Indian National Science Academy, Government of India (Grant No. NBS/2004/279), and Aligarh Muslim University, Aligarh, India (Grant No. Acad/D-280/AA) for the financial assistance provided to present part of this work at the 104th General Meeting of the American Society for Microbiology. The authors are grateful to M. Faisal for computer graphics and A. Arif for technical assistance.

Conflict of interest: No conflict of interest to declare.

References

1. Beg AZ, Ahmad I. Effect of *Plumbago zeylanica* and certain curing agents on multidrug resistant bacteria of clinical origin. *World J Microbiol Biotechnol* 2000;**16**:841–4.
2. Robin EH, Anril WM, Loeto M, Keith K. Nasopharyngeal carriage and antimicrobial resistance in isolates of *Streptococcus pneumoniae* and *Haemophilus influenzae* type b in children under 5 years of age in Botswana. *Int J Infect Dis* 1998;**3**:18–25.
3. Eichkoff TC. Antibiotics and nosocomial infections. In: Bennett JV, Brachman PS, editors. *Hospital infections*. 4th ed. Lippincott–Raven; 1998. p. 201–13.
4. Alvarez M, Tran JH, Chow N, Jacoby GA. Epidemiology of conjugative plasmid-mediated AmpC β-lactamases in the United States. *Antimicrob Agents Chemother* 2004;**48**:533–7.
5. Bauernfeind A, Chong Y, Lee K. Plasmid encoded AmpC beta-lactamases: how far have we gone 10 years after the discovery? *Yonsei Med J* 1998;**39**:520–5.
6. Shahid M, Malik A, Sheeba. Multidrug-resistant *Pseudomonas aeruginosa* strains harbouring R-plasmids and AmpC β-lactamases isolated from hospitalised burn patients in a tertiary care hospital of North India. *FEMS Microbiol Lett* 2003;**228**:181–6.
7. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disc method. *Am J Clin Pathol* 1966;**45**:493–6.
8. National Committee for Clinical Laboratory Standards. *Performance standards for antimicrobial disk susceptibility tests; approved standard M2-A7*. 7th ed. Wayne, PA, USA: National Committee for Clinical Laboratory Standards; 2000.
9. Livermore DM, Winstanley TG, Shannon KP. Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. *J Antimicrob Chemother* 2001; **48**(Suppl 1):87–102.
10. National Committee for Clinical Laboratory Standards. *Performance standards for antimicrobial susceptibility testing; ninth informational supplement (M100-S9)*. Wayne, PA, USA: National Committee for Clinical Laboratory Standards; 1999.
11. Shahid M, Malik A, Agrawal M, Singhal S. Phenotypic detection of extended-spectrum and AmpC β-lactamases by a new spot-inoculation method and modified three-dimensional extract test: comparison with conventional three-dimensional extract test. *J Antimicrob Chemother* 2004;**54**:684–7.
12. Davis LG, Dibner MD, Battey JF. Large scale alkaline lysis method of plasmid purification. *Basic methods in molecular biology*. New York: Elsevier; 1986. p. 99.
13. Shahid M, Malik A. Plasmid mediated amikacin resistance in clinical isolates of *Pseudomonas aeruginosa*. *Indian J Med Microbiol* 2004;**22**:183–5.
14. Portnoy DA, Moseley SL, Falkow S. Characterization of plasmids and plasmid associated determinants of *Yersinia enterocolitica* pathogenesis. *Infect Immunol* 1981;**31**:775–82.
15. Guerry P, Colwell RR. Isolation of cryptic plasmid deoxyribonucleic acid from Kanagawa positive strains of *Vibrio parahaemolyticus*. *Infect Immunol* 1977;**16**:28.
16. Shahid M, Malik A. MDR *Pseudomonas aeruginosa* in burn wounds: plasmid mediated resistance. *Bionotes* 2003:6–7.
17. Abigail S, Mathai E, Jesudason MV, John TJ. Ceftazidime resistance among *Klebsiella pneumoniae* in South India. *Indian J Med Res* 1995;**102**:53–5.
18. Vogel L, Jones G, Triep S, Koek A, Dijkshoorn L. RAPD typing of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens* and *Pseudomonas aeruginosa* isolates using standardized reagents. *Clin Microbiol Infect* 1999;**5**:270–6.
19. Jarlier V, Nicolas MH, Fournier G, Phillipon A. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. *Rev Infect Dis* 1988;**10**:867–78.
20. Ananthakrishnan AN, Kanungo R, Kumar A, Badrinath S. Detection of extended-spectrum beta lactamase producers among surgical wound infections and burns patients in JIPMER. *Indian J Med Microbiol* 2000;**18**:160–5.
21. Rao PS, Shivananda PG. Bacteraemia due to non-fermenting Gram-negative bacilli in immunocompromised patients. *Indian J Med Microbiol* 1993;**11**:95–9.
22. Veenu. Sikka R, Arora DR. Isolation and susceptibility pattern of non-fermenting Gram-negative bacilli from clinical samples. *Indian J Med Microbiol* 1998;**17**:14–8.
23. Ghatole M, Manthalkar P, Kandle S, Yemul V, Jahagirdar V. Correlation of extended-spectrum beta lactamases production with cephalosporin resistance in Gram-negative bacilli. *Indian J Pathol Microbiol* 2004;**47**:82–4.
24. Navon-Venezia S, Hammer Munz O, Schwartz D, Turner D, Kuzmenko B, Carmeli Y. Occurrence and phenotypic characteristics of extended-spectrum β-lactamases among members of the family *Enterobacteriaceae* at Tel-Aviv Medical Center (Israel)

and evaluation of diagnostic tests. *J Clin Microbiol* 2003;**41**: 155—8.

25. Winokur PL, Canton R, Casellas JM, Legakis N. Variations in the prevalence of strains expressing an extended-spectrum β-lactamase phenotype and characterization of isolates from Europe, the Americas, and the Western Pacific region. *Clin Infect Dis* 2001;**32**(Suppl 2):S94—103.
26. Coudron PE, Moland ES, Thomson KS. Occurrence and detection of AmpC β-lactamases among *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis* isolates at a veterans medical center. *J Clin Microbiol* 2000;**38**:1791—6.
27. Coudron PE, Hanson ND, Climo MW. Occurrence of extended-spectrum and AmpC β-lactamases in bloodstream isolates of *Klebsiella pneumoniae*: isolates harbor plasmid-mediated FOX-5 and ACT-1 AmpC β-lactamases. *J Clin Microbiol* 2003; **41**:772—7.
28. Manchanda V, Singh NP. Occurrence and detection of AmpC β-lactamases among Gram-negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Delhi, India. *J Antimicrob Chemother* 2003;**51**: 415—8.
29. Gazouli M, Tzouvelekis LS, Prinarakis E, Miriagou V, Tzelepi E. Transferable cefoxitin resistance in enterobacteria from Greek hospitals and characterization of plasmid-mediated group 1 β-lactamase (LAT-2). *Antimicrob Agents Chemother* 1996;**40**: 1736—40.