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MOLECULAR CHARACTERIZATION OF β-LACTAMASES FROM *E. COLI* AND *K. PNEUMONIAE* ISOLATED FROM A MULTICENTER TRIAL IN INDIA: BENCHMARK REPORT FROM THE MYSTIC PROGRAM

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MYSTIC
Meropenem Yearly Susceptibility Test Information Collection

INTRODUCTION

Longitudinal surveillance of resistance trends by multiple centers worldwide is one method by which the utility of antimicrobial agents against common pathogens can be assessed. There are several resistance mechanisms prominent in the world at this time, and the impact they have on the use of broad-spectrum and other antimicrobial agents differs according to a variety of environmental, socioeconomic and clinical factors. The diversity of one such resistance mechanism, the production of β-lactamases, is becoming more complex as extensive investigations continue to evaluate resistant bacteria at the biochemical and genetic level. It is important that not only the genotypic diversity be understood, but that the prevalence and the geographic variation of these enzymes be monitored.

Many geographic regions and countries around the world have committed resources to evaluate the resistance profiles of clinically significant bacteria. Reports have documented problems encountered by clinical practitioners in India regarding resistant bacteria. This study was done to determine the contemporary prevalence of resistance to broad-spectrum β-lactam agents particularly among Gram-negative pathogens in India. The intent of this presentation was to decipher the rates of β-lactamase production, including extended-spectrum β-lactamases (ESBLs), among *Escherichia coli* and *Klebsiella pneumoniae* from medical centers across India. The phenotypic and genotypic analysis of ESBL-like isolates was coupled with epidemiology methods to determine potential clonal dissemination within and between 10 hospitals located throughout India.

METHODS

10 laboratories in India including institutions in New Delhi (4 sites), Mumbai (2 sites), and 1 site each in Indore, Locknow, Bangalore, and Vellor were recruited to participate. Each was selected based on range of hospital size (≤399 to >1000 beds) and services provided including general medicine, surgical, and intensive care units. In addition, each site showed proficiency in the use and interpretation of the selected MIC methodology (Etest®, AB BIODISK, Solna, Sweden). Within the protocol sample, the laboratories were instructed to collect *E. coli* (EC) and *Klebsiella* spp. (KSP) isolates as they presented from clinically significant patient samples. This was done to establish the current prevalence of resistant phenotypes common within each institutional setting. Quality control strain (EC ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213) results were forwarded to the study coordinators (Mumbai, India and Iowa, USA) to determine the quality of the test results.

This network yielded 145 isolates of KSP and 132 EC, which were identified and tested for β-lactam susceptibility profiles at the local level. MIC determinations were read at 18 hours using NCCLS methods and interpretations (NCCLS, 2000).

All confirmed isolates presumptive of harboring ESBL enzymes by current NCCLS criteria (ceftazidime or cefotaxime MICs, ≥2 µg/ml) were forwarded to the international monitor for confirmation and additional strain analysis. This confirmation included Etest ESBL strips (ceftazidime or cefotaxime ± clavulanic acid) to phenotypically identify the presence or absence of an ESBL enzyme. Strains which seemed to be phenotypically related were characterized by pulsed-field gel electrophoresis and/or ribotyping (Riboprinter, Qualicon, Wilmington, DE). Other drug classes (fluoroquinolones and aminoglycosides) were tested to examine the extent of co-resistances among EC and KSP isolates with ESBL enzymes from India.

All genes encoding β-lactamases were aligned and generic primers designed for PCR analysis. In most cases PCR annealing was carried out at 48°C but gradient PCR was also used over a range of 38-60°C. Sequencing of BLs was carried out using DuPont Automated systems and analyzed using DNAstar.

RESULTS

- Among the strains tested, 74 isolates of EC and 53 KSP were available for confirmation; 71 and 48 strains, respectively, had an ESBL phenotype, and 65 and 46 strains, respectively, were confirmed ESBL producers (Table 1).

Table 1. Listing of organisms tested and their resistance features (127 isolates from India, 2000-2001)

Organism	No. tested	ESBL Phenotype[a]	ESBL Confirmed[b]	Comment
E. coli	74	71	65	2 susceptible controls were tested
Klebsiella spp.	53	48	46	3 susceptible controls were tested
Total	127	119	111	-

[a.] Phenotype = strains having a MIC of ≥2 µg/ml for aztreonam or ceftriaxone or ceftazidime.

[b.] Confirmed = strains with phenotype and demonstrating a ≥8-fold reduction of the MIC in the presence of clavulanic acid (2 µg/ml).

- Of the broad-spectrum agents, including β-lactams, aminoglycosides, and ciprofloxacin, tested against ESBL phenotypes of EC and KSP isolates, only meropenem retained excellent potency (MIC_{90}, 0.06 µg/ml) and activity (100.0% susceptible among these isolates) (Table 2). The only other compound with marginal activity (62.5-80.3% susceptible) was piperacillin/tazobactam.

Table 2. Antimicrobial activity of 10 broad-spectrum agents tested against 119 strains of *E. coli* and *Klebsiella* spp. having a positive ESBL resistance screening test[a]

Organism (no. tested)	Antimicrobial agent	MIC (µg/ml) 50%	90%	Range	% by category[a] Susceptible	Resistant
E. coli (71)	Meropenem	0.03	0.06	≤0.016-0.12	100.0	0.0
	Ceftazidime	>16	>16	2->16	7.0	52.1
	Ceftizoxime	>32	>32	4->32	8.5	81.7
	Ceftriaxone[b]	>32	>32	>32	0.0	100.0[b]
	Cefepime	>16	>16	8->16	2.8	84.5
	Aztreonam[b]	>16	>16	16->16	0.0	98.6[b]
	Piperacillin/ tazobactam	8	32	2->128	80.3	2.8
	Ciprofloxacin	>2	>2	>2	0.0	100.0
	Gentamicin	>8	>8	≤2->8	12.7	87.3
	Tobramycin	>8	>8	≤1->8	4.2	95.8
Klebsiella spp. (48)	Meropenem	0.03	0.06	≤0.016-1	100.0	0.0
	Ceftazidime	>16	>16	8->16	8.3	72.9
	Ceftizoxime	>32	>32	8->32	2.1	70.8
	Ceftriaxone[b]	>32	>32	32->32	0.0	95.8[b]
	Cefepime	>16	>16	1->16	8.3	85.4
	Aztreonam[b]	>16	>16	8->16	2.1	97.9[b]
	Piperacillin/ tazobactam	16	128	2->128	62.5	16.7
	Ciprofloxacin	2	>2	≤0.25->2	39.6	35.4
	Gentamicin	>8	>8	≤2->8	8.3	91.7
	Tobramycin	>8	>8	≤1->8	6.3	93.7

[a.] ESBL phenotype as defined by the NCCLS [23]. A total of 111 strains (93.3%) had an enzyme inhibited by clavulanic acid (4 µg/ml), a positive confirmation result.

[b.] The most enzyme-sensitive substrates for ESBL recognition in India.

- After ruling out phenotypically and epidemiologically related isolates (clonal dissemination), 23 strains of EC and 20 KSP isolates were analyzed using PCR to detect 5 different enzymes (Tables 3 and 4). 9 centers had EC and 8 centers had KSP isolates with positive PCR products for the enzymes analyzed.
- For each species (group), 1 to 5 enzymes (including a novel TEM in EC; first isolation of CMY-7 in KSP) were detected and the co-resistances to ciprofloxacin and gentamicin were very common in these isolates.
- Phenotypically related strains were detected in 3 institutions (EC) and two institutions (KSP) suggesting intra-hospital spread of ESBL isolates.

Table 3. Phenotypic and genotypic analysis of 23 ESBL-producing isolates of *E. coli* from Indian medical centers

Isolate #	Enzymes	ESBL phenotype/Etest	TEM-1	SHV-1	CTX-M	CMY	OXA-1[a]	OXA-2[a]	Co-R[b]	Clonal cluster	Ribotype
A4/7	3	+/+	+	-	+	-	+	-	GC	N	263.1
A4/8	4	+/+	+	-	M15	-	+	+	GC	N	243.2
A4/11	3	+/+	+	-	+	-	+	-	GC	N	-
B4/6	3	+/-	+	-	M15	-	-	+	GC	Y(2)	253.7
B4/12	4	+/+	+	+	+	-	+	-	GC	N	-
B5/14	3	+/+	+	-	+	-	+	-	GC	N	519.3
C4/3	2	+/+	+	-	+	-	-	-	C	N	283.6
C4/12	2	+/+	+	-	+	-	-	-	C	N	252.1
D4/11	ND	+/+	ND[c]	ND	ND	ND	ND	ND	GC	Y(2)	243.2
D4/12	4	+/+	+	-	M15	-	+	+	GC	N	242.5
E4/1	1	+/+	+	-	-	-	-	-	GC	N	-
E4/4	1	+/+	+	-	-	-	-	-	GC	N	519.3
E4/6	3	+/+	NEW[d]	-	M15	-	-	+	GC	N	492.1
G4/10	4	+/+	-	+	M15	-	+	+	GC	N	519.3
G4/12	5	+/-	+	-	M15	CMY6	OXA-1	+	GC	N	-
G4/13	3	+/+	+	-	M15	-	-	+	GC	N	242.5
I4/3	3	+/+	+	ND	M15	-	+	-	C	N	243.2
I4/9	3	+/+	+	+	-	-	-	+	GC	N	242.5
I4/13	4	+/+	+	+	+	-	+	-	GC	N	263.1
F4/3	3	+/+	+	-	+	-	+	-	C	N	-
F5/10	4	+/+	+	+	+	-	+	-	G	N	-
H4/5	4	+/+	+	-	+	-	+	+	GC	Y(2)	243.2
H4/8	3	+/+	+	-	M15	-	+	-	GC	N	263.2

[a.] Two OXA-primer sets.

[b.] G = gentamicin-resistant and C = ciprofloxacin-resistant.

[c.] ND = not done due to the potential genetic relatedness with a previously analyzed isolate.

[d.] NEW = novel TEM sequence detected.

Table 4. Phenotypic and genotypic analysis of 20 ESBL-producing isolates of *Klebsiella* spp. from Indian medical centers

Isolate #	ESBL phenotype/Etest	TEM-1	SHV-1	CTX-M	CMY	OXA-1[a]	OXA-2[a]	Co-R[b]	Clonal cluster	Ribotype
A5/3	+/+	+	ND[c]	+	-	+	+	GC	N	-
A5/4	+/+	+	+	+	-	-	-	-	N	-
A5/7	+/+	+	+	+	CMY-7	+	+	GC	N	-
B5/11(KOX)	+/+	+	-	-	-	-	-	GC	Y(2)	283.8
B5/16	+/-	ND	+	-	CMY-4	-	+	GC	N	-
C5/5	+/+	+	+	+	-	+	-	GC	N	448.2
C5/7	+/+	+	+	M15	-	+	+	GC	N	-
C5/8	+/+	+	ND	+	-	+	-	GC	N	283.5
D5/4	+/+	+	+	+	-	+	-	G	N	-
D5/12	+/-	ND	+	M15	CMY-4	+	+S	GC	N	-
E5/14	+/+	+	+	M15	-	+	-	GC	N	284.4
E /17	+/+	+	+	M15	-	-	+	G	N	-
E /19	+/+	+	+	M15	-	+	+	GC	N	356.7
G5/2	+/+	+	+	+	-	+	-	GC	N	1795.3
G5/6	+/+	+	+	M15	-	-	-	GC	N	1795.4
G5/11	+/+	ND	+	M15	-	+	+	GC	N	1795.5
I5/5	+/+	+	+	+	-	-	-	GC	Y(3)	79.2
F5/6	+/+	+	ND	-	-	-	-	GC	N	448.2
F5/10	+/+	+	+	+	-	+	-	G	N	-
F5/11	+/+	+	+	+	-	+	-	GC	N	283.1

[a.] Two OXA-primer sets.

[b.] G = gentamicin-resistant and C = ciprofloxacin-resistant.

[c.] ND = not done due to the potential genetic relatedness with a previously analyzed isolate.

CONCLUSIONS

- **EC-harboring ESBL enzymes are very common in Indian hospitals with TEM, CTX-M and OXA enzymes being common.**
- **ESBL enzymes (CTX-M and others) are also very common in KSP, and CMY enzymes may be becoming more prevalent in this species.**
- **Strains of EC and KSP are quite resistant to β-lactams due to enzyme activities ubiquitous in Indian hospitals and co-resistance to aminoglycosides and fluoroquinolones was nearly complete.**
- **Meropenem was the only active antimicrobial agent that retained complete coverage for these resistant pathogens.**
- **Clonal dissemination of these resistant phenotypes is of great concern and continued surveillance will be critical to the limitation of their spread.**

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