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Identification of TEM-26 β-Lactamase Responsible for a Major Outbreak of Ceftazidime-Resistant *Klebsiella pneumoniae*

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An epidemic of nosocomial ceftazidime-resistant *Klebsiella pneumoniae* was correlated with production of a ceftazidime-hydrolyzing enzyme with an isoelectric point of 5.6 (BMH-1). BMH-1 was encoded on a large transferable plasmid conferring multiple antibiotic resistance. The gene that encodes BMH-1 was identical to the gene that encodes the TEM-26 extended-spectrum β-lactamase.

Organisms that produce enzymes capable of hydrolyzing extended-spectrum cephalosporins were first reported from Europe (8, 12) but have been reported with increasing frequency in the United States (14, 16). Outbreaks of ceftazidime-resistant *Klebsiella pneumoniae* have been reported in a Massachusetts chronic-care facility (17), a pediatric hospital in California (11), and more recently in Flushing, New York (10). In each outbreak, the occurrence of ceftazidime-resistant organisms was correlated with increased usage of ceftazidime. The epidemic described in this report involved *K. pneumoniae* possessing multiple β-lactamases. In these isolates, a β-lactamase with an isoelectric point of 5.6 was shown to be responsible for ceftazidime resistance.

A total of 436 ceftazidime-resistant *K. pneumoniae* isolates were obtained during a 19-month period from October 1988 to March 1990 from 155 patients of The New York Hospital Medical Center of Queens, formerly Booth Memorial Medical Center (10). These organisms were isolated and identified by the hospital clinical microbiology laboratory. Ceftazidime resistance was defined as a zone of inhibition of growth corresponding to 15 mm or less by the Kirby-Bauer disk diffusion susceptibility method according to the National Committee for Clinical Laboratory Standards guidelines at that time (10a). Antibiotics were supplied from their manufacturers.

The β-lactamase with a pI of 5.6 (BMH-1) was purified from both the clinical isolate *K. pneumoniae* 223 and from the transformant *Escherichia coli* DH5α containing plasmid pCLL2305. *K. pneumoniae* 223 was grown for 4 h in Trypticase soy broth (TSB) from a culture grown overnight. Harvested cells were broken in 0.2 M sodium acetate, pH 5.5, using a freeze-thaw procedure (5). The supernatant from the extract was dialyzed overnight in 0.05 M phosphate buffer, pH 6.5, and eluted from a column of CM-Sephadex in the same buffer. BMH-1 was eluted after the void volume; a β-lactamase of pI 7.6 (BMH-2) appeared after at least 1 column volume of buffer had been washed through the column. An extract containing BMH-2 prepared by freeze-thawing was also purified from *K. pneumoniae* 227, yielding an enzyme preparation with higher specific activity for kinetic studies. Enzyme activity was eluted from Sephadex G-75 in 0.05 M phosphate buffer, pH 7.0. BMH-1 was also isolated from *E. coli* DH5α containing pCLL2305, using an extract prepared by freeze-thawing that was further purified by using a boronic acid affinity column (4). The identities of all β-lactamases were confirmed by isoelectric focusing (9) on PAGplates, pH range 3.5 to 9.5, using an LKB Multiphor electrophoresis unit. Preparations of BMH-1 and BMH-2 purified by CM-Sephadex chromatography contained a single β-lactamase activity but were less than 50% homogeneous by protein staining. BMH-1 purified by affinity chromatography yielded a single β-lactamase of pI 5.6 that was greater than 90% homogeneous by protein staining.

Hydrolysis studies were performed spectrophotometrically as described previously (4, 14), using initial reaction rates obtained with at least five different substrate concentrations. All assays were conducted with a single purified enzyme activity. Three different preparations of BMH-1 were used to determine the kinetic parameters V_{max} and K_m (12), calculated by the program ENZPACK (Elsevier). The coefficient of variation for ceftazidime relative V_{max} values was 7.2%. Inhibition studies were performed by incubating clavulanic acid or tazobactam with purified enzyme for 5 min before the addition of 1.0 mM cephaloridine (14). The concentration required for inhibition of 50% of the enzyme activity was obtained graphically.

Transconjugants were generated by mixing donor *K. pneumoniae* and recipient *E. coli* SC 12759 on diagnostic sensitivity test agar (Oxoid Ltd., Basingstoke, Hampshire, England). After overnight incubation at 37°C, bacterial growth was scraped from the agar and plated on diagnostic sensitivity test agar containing ceftazidime at 10 μg/ml and nalidixic acid at 100 μg/ml. Plasmid DNA was isolated by the rapid alkaline lysis technique of Portnoy et al. (13). DNA was submitted to electrophoresis in 0.7% agarose at 12 V/cm and visualized by staining with ethidium bromide. An oligonucleotide with the sequence 5′-AACGCGAGGAC-3′ specific for TEM-type enzymes was used to probe the BMH-1 gene with the hybridization conditions described previously (14). The β-lactamase gene encoding the pI 5.6 enzyme was localized to a 4.2-kb *Bam*HI fragment that was cloned into pCLL2300, a kanamycin-resistance-conferring cloning vector (15). The resulting plasmid, pCLL2305, was transformed into DH5α cells (Be-

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TABLE 1. Isoelectric focusing patterns of β-lactamases from clinical isolates originally identified as ceftazidime-resistant *K. pneumoniae* strains

Isolate	Presence of band containing enzyme with isoelectric point[a]:					Isoelectric focusing pattern in:		Ceftazidime resistance in 1992[b]
	5.4	5.6	6.4	7.2	7.6	1990 (initial)	1992	
219	+	+	+			A	NT[c]	NT
220	+	+	+		+	B	NT	NT
221	+	+	(+)			A	G	R
223		+			+	C	C	R
224	(+)	(+)	(+)		+	B	E	S
225	+	+	+		+	B	NT	NT
226	(+)	(+)	(+)	+		D	H	S
227[d]					+	E	E	S
228	+	+	(+)			A	G	R
229	+	(+)			+	F	I	S
062[e]	+	+	+		+	NT	A	R

[a] +, enzyme band present; (+), enzyme band lost when stored in the absence of ceftazidime.
[b] NT, not tested; R, resistant; S, susceptible.
[c] NT, not tested.
[d] Isolate not resistant to ceftazidime when the initial extract was prepared.
[e] Isolated July 1991.

thesda Research Laboratories, Inc.), and the DNA sequence of the TEM gene within the 4.2-kb fragment was determined. At least one strand of the entire coding sequence of the TEM gene was sequenced. A set of oligonucleotides complementary to TEM-coding sequences was selected so that they did not anneal to regions of the gene corresponding to base pair changes associated with extended-spectrum TEM gene DNA sequences (14). DNA sequence analysis was performed on double-stranded plasmid DNA with a Sequenase kit (United States Biochemical) according to the manufacturer's instructions.

Eleven randomly selected ceftazidime-resistant *K. pneumoniae* isolates were examined in detail. Two isolates lost their ceftazidime resistance during the first passage of the organism in the absence of ceftazidime. After isoelectric focusing of the extracts, BMH-1 with a pI of 5.6 was common to all the ceftazidime-resistant isolates (Table 1). Isolates initially examined in 1990 often produced three or four β-lactamases. However, when a set of these original isolates was reexamined after being stored without ceftazidime, a number of the β-lactamase bands no longer appeared. Those isolates that lost BMH-1 became fully susceptible to ceftazidime. A more recent isolate, strain 062 from July 1991, demonstrated a typical isoelectric focusing pattern A, with high levels of resistance to ceftazidime and a faint band with a pI of 7.6.

As seen in Table 2, benzylpenicillin, cephaloridine, and ceftazidime were hydrolyzed by BMH-1 at almost equivalent rates. Ceftazidime was hydrolyzed much faster than cefotaxime, and aztreonam was hydrolyzed at an intermediate rate. The kinetic parameters for BMH-1, therefore, were similar to those observed for the TEM-26 β-lactamase (11). BMH-1 was inhibited by clavulanic acid and tazobactam with 50% inhibitory concentrations of 30 and 34 nM, respectively. Biochemically, BMH-1 fulfills the criteria for an extended-spectrum β-lactamase of group 2b′ defined by Bush (2).

The BMH-2 enzyme with an isoelectric point of 7.6 was compared with SHV-1 and SHV-2 and with a group of the cloxacillin-hydrolyzing β-lactamases, enzymes with pI values ranging from 7.4 to 7.7 (3). Because BMH-2 did not hydrolyze the extended-spectrum cephalosporins, it could not be SHV-2. BMH-2 also hydrolyzed cloxacillin at a negligible rate in contrast to the cloxacillin-hydrolyzing enzymes with pI values of 7.4 to 7.7, which had rates greater than 200. The lone exception was OXA-4, with a relative hydrolysis rate of 33 for cloxacillin, but a similar rate of 32 for cefotaxime. Thus, BMH-2 is most likely the common SHV-1 broad-spectrum β-lactamase of group 2b of Bush (2).

Plasmid profiles were determined for five representative isolates. All five possessed a large (approximately 150-kb) transferable plasmid. Strain 223 also contained an 82-kb plasmid; strain 226 contained an additional plasmid of 90 kb and a third plasmid of >200 kb. All these isolates were

TABLE 2. Kinetic parameters for BMH-1 and BMH-2 compared with TEM-26 and SHV-1 β-lactamases

Antibiotic	BMH-1 (pI 5.6)		TEM-26 (pI 5.6), relative V_{max}[a]	BMH-2 (pI 7.6)		SHV-1 (pI 7.6), relative V_{max}[b]
	K_m (μM)	Relative V_{max}		K_m (μM)	Relative V_{max}	
Cephaloridine	140	100	100	280	100	100
Cefotaxime	43	5.0	6.3	NS[c]	≤0.4	0.38
Ceftazidime	89	125	140	NS	≤0.1	0.04
Aztreonam	70	34	41	630	0.66	0.79
Benzylpenicillin	8.0	140	83	14	240	210
Cloxacillin	ND[d]	ND	14	NS	≤10	1.7

[a] Data taken from reference 11, except for recalculated ceftazidime data.
[b] Data taken from reference 2.
[c] NS, not significant (rate was too slow to obtain reliable rates).
[d] ND, not determined.

TABLE 3. Sequence differences between cloned DNA sequences encoding TEM-26 β-lactamase compared with a TEM-1 DNA sequence

Nucleotide[a]	Nucleotide (amino acid[b]) in β-lactamase[c]:		
	TEM-1 from Tn2	TEM-26	
		From pJPQ101	From pCLL2305
226	T (Phe-8)	T (Phe-8)	T (Phe-8)
436	T (Gly-78)	T (Gly-78)	T (Gly-78)
512	G (Glu-104)	**A (Lys-104)**	**A (Lys-104)**
604	T (Ala-134)	T (Ala-134)	T (Ala-134)
692	C (Arg-164)	**A (Ser-164)**	**A (Ser-164)**

[a] Nucleotide numbering of Sutcliffe (19).
[b] Amino acid numbering of Ambler et al. (1).
[c] The data for TEM-1 are taken from references 5 and 6. TEM-26 from pJPQ101 was originally isolated from *K. pneumoniae* KPS-1, and TEM-26 from pCLL2305 was originally isolated from *K. pneumoniae* 221. Substitutions are shown in boldface type.

resistant to ampicillin, mezlocillin, tobramycin, gentamicin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. Three strains were shown to cotransfer resistance to these antibiotics. Because two of the representative isolates were also resistant to nalidixic acid, we were unable to find an appropriate marker for counterselection. DNA hybridization analysis using the oligonucleotide 5′-AACGCGAGCGAC-3′ indicated that the 150-kb plasmid carried a TEM-type β-lactamase.

The nucleotide sequence for the β-lactamase gene on plasmid pCLL2305 appeared to be derived from a TEM-1 gene, with two base changes occurring at positions 512 and 692 (Table 3). The resulting amino acid changes of Lys-104 for Glu-104 and Ser-164 for Arg-164 in TEM-1 are the same as those reported for the TEM-26 sequence from pJPQ101 (11). This gene is probably from a Tn*2* transposon (6, 7).

On the basis of sequence information, it is clear that the BMH-1 sequence is identical to that described for the TEM-26 enzyme (11). However, biochemically, the rate of hydrolysis for ceftazidime was significantly lower than that reported in the initial TEM-26 description. Each TEM-26 enzyme (BMH-1 and KPS-1) was analyzed in the same laboratories at least three times. BMH-1 was examined both from a clinical isolate and from a transconjugant to determine whether there might be unique processing of the enzyme in one strain. The data for ceftazidime hydrolysis were highly consistent for all BMH-1 preparations. This discrepancy could be resolved by recalculating the original TEM-26 (KPS-1) data using only the highest substrate concentrations in the range (100 to 150 μM): a lower relative V_{max} value was obtained (Table 2). Even with this lowered V_{max}, however, the TEM-26 enzyme remains the extended-spectrum TEM β-lactamase with the highest hydrolysis rate for ceftazidime.

Three different centers in the United States have now reported epidemics of ceftazidime-resistant *K. pneumoniae* that have been associated with the TEM-26 β-lactamase (10, 11, 18). It is curious to note that all these outbreaks originated in 1988 but were located on opposite coasts of the United States: Stanford, Calif. (11), Youville, Mass. (16), and Flushing, N.Y. (10). The amino acid changes associated with TEM-26, Lys-104 and Ser-164, are functionally equivalent to those found in the commonly identified TEM-10 enzyme, Ser-164 and Lys-240 (11, 14, 18). At this time, the TEM-10 and TEM-26 β-lactamases are the most frequently identified extended-spectrum TEM enzymes in the United States, in contrast to France where TEM-3 and TEM-5 are the prevalent extended-spectrum TEM β-lactamases (12). In all cases, however, the occurrence of these enzymes was associated with increased use of ceftazidime. The observation that some of the resistant isolates spontaneously lost several β-lactamases when stored in the absence of ceftazidime corresponded to the drop in the number of resistant *K. pneumoniae* isolated from the hospital when the antibiotic was put on restricted use. For this reason, it is important to use the most potent antibiotics only when necessary and to be ready to withdraw them upon the development of resistance.

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