JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1994, p. 2481–2486
0095-1137/94/$04.00+0

Vol. 32, No. 10

Detection of Resistance Due to Inducible β-Lactamase in *Enterobacter aerogenes* and *Enterobacter cloacae*

THOMAS W. HUBER* AND J. SCOTT THOMAS

Department of Pathology and Laboratory Medicine and Department of Medical Microbiology and Immunology, Texas A&M College of Medicine, Olin E. Teague Veterans' Center, Temple, Texas 76504

Received 5 May 1994/Returned for modification 30 June 1994/Accepted 26 July 1994

Thirty-six of 36 strains of *Enterobacter cloacae* and *E. aerogenes* with inducible β-lactamase developed resistance when cefoxitin (inducer) was added to cefuroxime disks. Constitutive β-lactamase producers (n = 23) were all resistant to cefuroxime. Cefuroxime resistance correlated with the amount of induced or constitutive β-lactamase. Cefuroxime was a better indicator of induced resistance than cefamandole, cefazolin, cephalothin, ceftriaxone, cefotaxime, ticarcillin with or without clavulanic acid, or cefotetan. Induction by addition of cefoxitin to disks occasionally reduced zone sizes but not enough to change interpretations for ceftazidime, ceftizoxime, aztreonam, cefoperazone with or without sulbactam, and piperacillin with or without tazobactam. Most enterobacters were resistant to cefmetazole. The cefoxitin inducer-cefuroxime indicator method can be used in routine clinical laboratories to detect latent resistance due to chromosomally mediated inducible β-lactamase in enterobacters.

Enterobacters and many other gram-negative bacilli carry a gene for a chromosomally encoded β-lactamase which can be induced by certain antibiotics, amino acids, or body fluids (4, 6). It is a concern that organisms harboring genes for inducible β-lactamases may show false susceptibility if tested in the uninduced state (14). Therapeutic failures with antibiotics to which organisms were initially susceptible have been reported (2, 13, 16, 17). Several methodologies for laboratory detection of inducible resistance have been suggested. Sanders and Sanders (15) proposed a disk approximation test in which cefoxitin (inducer) disks were placed adjacent to antibiotic test disks. If the test zones were flattened by ≥4 mm by the adjacent inducer disk, the organism was said to have inducible resistance. Recently, Nadal and von Graevenitz (10) suggested routine use of the disk approximation test employing cefoxitin disks dispensed at standard distances and that a ≥1-mm truncation of zones indicates resistance. Addition of cefoxitin to microtiter dilution (9, 12) and agar dilution (8) susceptibility tests has had limited success. To establish a standardized, reproducible, and therefore interpretable method, we devised a method for measuring induced resistance by using the standard disk diffusion susceptibility test method modified by adding the inducer to test disks.

MATERIALS AND METHODS

Selection of isolates. Thirty-one enterobacter strains were collected as consecutive nonduplicate clinical isolates at the Olin E. Teague Veterans' Center microbiology laboratory from December 1989 to March 1990. Twenty-nine enterobacter strains from blood, cerebrospinal fluid, intravenous sites, and wounds were collected at the St. Louis University Hospital, St. Louis, Mo., from October 1991 to November 1992. *Enterobacter cloacae* 55 and 55M were received from Christine Sanders, Creighton University, Omaha, Nebr., and used as controls for inducible and constitutive (stably derepressed) β-lactamase producers. Significant information about each of the 62 organisms is summarized in Table 1. Organisms were stored at −50°C in Trypticase soy broth glycerol vials (Pro-Lab Diagnostics, Round Rock, Tex.).

Growth of inocula. A bead from frozen vials was roll inoculated onto MacConkey's agar to revive the organisms. All organisms were subcultured at least once prior to susceptibility testing.

Induction of β-lactamase. Overnight Trypticase soy broth cultures were inoculated 1:100 into 10 ml of Trypticase soy broth and incubated on a reciprocal shaker (200 rpm) at 35°C. Cefoxitin was added to 3-h Trypticase soy broth cultures to achieve a final concentration of 0, 5, or 30 μg/ml, and incubation was continued to 4 h. Organisms were harvested from 4-h cultures by centrifugation at 2,000 × g for 10 min, washed three times in 10 ml of 0.1 M phosphate-buffered saline (pH 7.3), and resuspended in phosphate-buffered saline to 1/10 of the original volume.

Sonic disruption of organisms. Washed organisms were sonically disrupted with a W-375 Cell Disruptor (Heat Systems Ultrasonics, Inc., Plainview, N.Y.) or a Fisher 300 Sonic Dismembrator (Imaging Products International, Chantilly, Va.) at maximum output for 2 min. Materials were sonicated in an ice bath to prevent thermal denaturation of enzymes. Sonicates were sedimented at 2,000 × g for 15 min. The supernatant fluid and sediments were separated and frozen at −20°C pending protein and β-lactamase assay.

Protein assay. Protein concentration was determined with a bicinchoninic acid assay (Sigma, St. Louis, Mo.) modified for testing on a Technicon RA-1000 autoanalyzer. The following parameters were determined to be optimal for the assay: TYPE, 1; %SMP VOL, 60; FILTER POS, 5 WL550; DELAY, 0 30; INCUBATION, 8 30; %RGT VOL, 65; UNITS, 15 μg/ml; UNIT FAC, 1.0000; DECIMAL PT, 0; RBL LOW, 0.000; RBL HI, 3.000; RANGE LOW, 0; RANGE HI, 2500; NORMAL LO, 0; NORMAL HI, 2000; SLOPE, 1.000; INTERCPT, 0.0000; LIN FACT, 5.00; 1ST LIM, 0.0453. Protein concentrations ranging from 25 to 4,000 μg/ml could be measured.

β-Lactamase assay. β-Lactamase activity was detected with

* Corresponding author. Mailing address: Medical Microbiology and Immunology, Texas A&M University College of Medicine, Olin E. Teague Veterans' Center, 1901 South First Street, Temple, TX 76504. Phone: (817) 771-4545. Fax: (817) 771-3098. Electronic mail address: THUBER@summa.tamu.edu.

TABLE 1. Source, classification, and inducible resistance of organisms

Strain	Source	Type	β-Lactamase activity (U/mg of protein)		Induced resistance to[a]:
			Basal	Induced	
E. cloacae U97	Urine	L[b]	0	190	
E. cloacae U66	Urine	L	17	744	CF
E. aerogenes 1088A	Wound	L	23	0	
E. cloacae O58	Sputum	I	62	6,323	CXM, MA, TIC, TIM
E. cloacae O102	Sputum	I	31	3,270	CXM
E. cloacae O57	Foot	I	28	2,903	CXM, MA
E. cloacae O25	Unknown	I	110	1,457	CXM, MA
E. cloacae U101	Urine	I	85	7,750	CXM, MA
E. cloacae U67	Urine	I	15	1,370	CXM
E. cloacae U61A	Urine	I	191	9,236	CXM, MA, CTT, TIM
E. cloacae U19	Urine	I	111	3,841	CXM
E. cloacae U13	Urine	I	15	3,394	CXM, MA, CTX, TIM
E. cloacae O60	Sputum	I	26	2,343	CXM, CRO
E. cloacae U57	Urine	I	17	2,126	CXM, MA
E. cloacae O6	Knee	I	19	3,174	CXM, MA
E. cloacae U7	Urine	I	87	1,516	CXM, MA
E. cloacae O34	Leg	I	20	3,050	CXM, CRO, CTX, TIM, CTT
E. cloacae O20	Groin	I	32	3,882	CXM, MA, CTT, CRO
E. cloacae U21	Urine	I	42	2,686	CXM, CF
E. cloacae U60	Urine	I	58	2,746	CXM
E. cloacae O30	Hand	I	84	2,604	CXM, CZ
E. cloacae 55	C. Sanders	I	31	2,014	CXM, MA
E. cloacae 999	Blood	I	46	1,692	CXM
E. cloacae 2271	Blood	I	21	6,105	CXM, MA, TIM
E. cloacae 767	i.v.[c]	I	87	6,275	CXM, MA
E. cloacae 408	Skin	I	8	4,531	CXM, MA, TIC
E. cloacae 232	Tissue	I	24	6,287	CXM, MA
E. cloacae 230	i.v.	I	146	11,750	CXM, MA, TIM, CTT
E. aerogenes O39	Sputum	I	25	2,346	CXM, MA
E. aerogenes O26	Sputum	I	67	6,885	CXM, CZ
E. aerogenes O23	Sputum	I	120	5,157	CXM
E. aerogenes U27	Urine	I	36	2,133	CXM, MA
E. aerogenes U24	Urine	I	27	1,814	CXM, CZ
E. aerogenes U40	Urine	I	67	3,453	CXM
E. aerogenes 2158	Blood	I	92	2,788	CXM, MA
E. aerogenes 2051-2	CSF[d]	I	0	5,361	CXM, TIM
E. aerogenes 369	CSF	I	50	3,541	CXM, CZ
E. aerogenes 292	CSF	I	62	4,249	CXM, CZ
E. aerogenes 273	CSF	I	59	3,219	CXM, CZ
E. cloacae U61B	Urine	C	12,014	58,124	
E. cloacae U6	Urine	C	5,648	3,241	
E. cloacae U1	Urine	C	275	3,090	ATM, CAZ
E. cloacae O19	Elbow	C	2,332	5,118	
E. cloacae 55M	C. Sanders	C	6,951	18,323	
E. cloacae 1759	Blood	C	839	4,630	
E. cloacae 1758	Blood	C	841	3,161	
E. cloacae 1006	i.v.	C	3,515	6,576	
E. cloacae 886	i.v.	C	4,325	9,167	
E. cloacae 845	i.v.	C	7,520	8,773	
E. cloacae 586	i.v.	C	1,968	7,894	
E. cloacae 458	Blood	C	2,347	6,364	
E. cloacae 457	Blood	C	8,646	7,293	
E. cloacae 425	Tissue	C	4,783	2,984	
E. aerogenes O16	Sputum	C	205	10,000	CRO, CAZ, TIC
E. aerogenes 2051-1	CSF	C	1,982	9,724	
E. aerogenes 2034-2	CSF	C	1,441	1,531	
E. aerogenes 2034-1	CSF	C	2,871	14,803	
E. aerogenes 1088B	Wound	C	1,982	8,485	
E. aerogenes 517-2	CSF	C	10,732	23,489	
E. aerogenes 517-1	CSF	C	5,443	17,808	
E. aerogenes 422	Unknown	C	2,120	5,692	
E. aerogenes 196	Blood	C	1,381	3,956	

[a] Abbreviations: CF, cephalothin; CXM, cefuroxime; MA, cefamandole; TIC, ticarcillin; TIM, ticarcillin-clavulanic acid; CTT, cefotetan; CRO, ceftriaxone; CTX, cefotaxime; CZ, cefazolin; ATM, aztreonam; CAZ, ceftazidime.

[b] Types: L, low producer; I, inducible; C, constitutive. Strain designations with no prefix letter were collected at the St. Louis University Hospital, except 55 and 55M, which were received from C. Sanders, Creighton University School of Medicine.

[c] i.v., intravenous catheter.

[d] CSF, cerebrospinal fluid.

10^{-4} M CENTA (Calbiochem, San Diego, Calif.) (7) as the substrate and a user-defined enzyme rate assay with a Technicon RA-1000 autoanalyzer. The following instrument parameters were used: TYPE, 0; %SMP VOL, 60; FILTER POS, 3 WL405; DELAY, 0 15; %RGT VOL, 76; UNITS, 3 U/liter; UNIT FAC, 1.0000; DECIMAL PT, 0; RBL LOW, 0.000; RBL HI, 3.000; RANGE LO, 25; RANGE HI, 5000; CAL FACTOR, 2466.28; RGRT RATE, 0.0009; NORMAL LO, 25; NORMAL HI, 250; SLOPE, 1.000; INTERCPT, 0.0000; C1*10E-6, 9999.00; C2*10E-6, 9999.00; D1*10E-6, 9999.00; DELTA no., 0.020. The measured threshold of detection was 5 U, but precision was achieved in the range of 25 to 250 U. Out-of-range samples (>250 U of activity) were appropriately diluted prior to the final assay. A unit of β-lactamase was defined as the amount of enzyme required to degrade 1 μmol of CENTA per min at 37°C and pH 7.3. Standard concentrations ranging from 25 to 200 U of type I penicillinase from *Bacillus cereus* (Sigma) were used to verify performance on each test day.

Specific β-lactamase activity. Specific β-lactamase activity was defined as units of β-lactamase per milligram of protein.

Selection of indicator antibiotic. Standardized suspensions prepared from colonies of enterobacters in the stationary phase were streaked onto Mueller-Hinton agar in accordance with standard disk diffusion susceptibility procedures (11). Disks containing cefuroxime, cephalothin, cefamandole, cefazolin, ceftriaxone, cefotaxime, ceftazidime, ceftizoxime, cefotetan, cefmetazole, cefoperazone, cefoperazone-sulbactam, aztreonam, ticarcillin, ticarcillin-clavulanic acid, piperacillin, and piperacillin-tazobactam were applied to duplicate plates to determine which antibiotic most effectively demonstrated inducible resistance. Thirty micrograms of cefoxitin in 5 μl of sterile water was then delivered by micropipette with a sterile, disposable tip to disks on the induction plates. Disks on control plates received 5 μl of sterile water. Zones were measured and interpreted by using standard methods (11). Cefoperazone-sulbactam disks obtained from Pfizer Laboratories, New York, N.Y., contained 75 μg of cefoperazone and 30 μg of sulbactam. Interpretations (susceptible, ≥21 mm; resistant ≤15 mm) were done as described by Barry et al. (1). Piperacillin-tazobactam disks with 100 and 10 μg, respectively, now marketed as Zosyn, were obtained from Lederle Laboratories, Pearl River, N.Y.

Determination of inducer concentration. Each inoculum was prepared and swabbed onto 100-mm-diameter Mueller-Hinton agar plates in accordance with standard disk diffusion methods (11). Replicate disks were dispensed onto induction plates and control plates. Cefoxitin at 0, 1, 5, 10, 20, or 30 μg in 5 μl of sterile water was dispensed onto inducer plate disks. No cefoxitin was added to disks on control plates. Zone sizes were measured and interpreted by using standard methods (11).

RESULTS

Selection of an antimicrobial agent as an indicator of resistance due to inducible β-lactamase. The effect of inducible β-lactamase on 17 different antimicrobial agents was evaluated by using disks with and without addition of 30 μg of cefoxitin and 36 enterobacter strains with inducible enzymes. The data in Fig. 1 show the antimicrobial agents to which resistance could be induced. Cefuroxime was the best indicator of cefoxitin-induced resistance. Thirty-five of 36 enterobacter isolates with inducible β-lactamase were susceptible or intermediately susceptible to cefuroxime control disks but resistant when 30 μg of cefoxitin was added to the disks prior to incubation. One isolate, *E. aerogenes* U24, gave zone sizes of 23 (susceptible) and 16 (intermediately susceptible) mm when

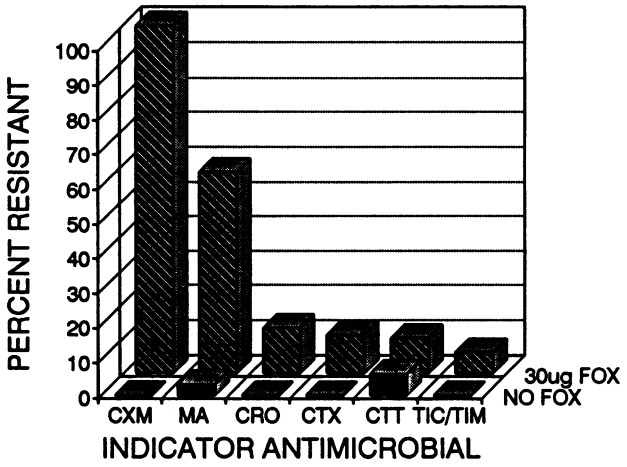

FIG. 1. Effect of cefoxitin (FOX) on disk diffusion interpretations for 36 enterobacters with inducible β-lactamase. Antimicrobial agents with indicator potential: cefuroxime (CXM), cefamandole (MA), ceftriaxone (CRO), cefotaxime (CTX), cefotetan (CTT), and ticarcillin without and with clavulanic acid (TIC/TIM). The results obtained with ticarcillin with and without clavulanic acid were identical.

tested against cefuroxime without and with added cefoxitin, respectively. Cefamandole was the next best indicator but appeared to be more refractory to degradation by induced β-lactamase. Although induction sometimes reduced the zone sizes obtained with other effective antibiotics, interpretations usually remained in the susceptible range. The identical results obtained for ticarcillin and ticarcillin-clavulanic acid reflect the inability of clavulanic acid to inhibit inducible β-lactamase. Antibiotics which were ineffective indicators of induction are not shown in Fig. 1. Cefazolin, cephalothin, and cefmetazole were ineffective indicators because the enterobacter isolates tested were too often resistant prior to induction. Susceptibility to ceftazidime, ceftizoxime, aztreonam, cefoperazone with and without sulbactam, and piperacillin with and without tazobactam was constant regardless of induction.

Illustration of induced resistance. Figure 2 shows the disk diffusion zone of a typical inducible organism exposed to cefuroxime and the resistance which emerged upon addition of a solution containing 30 μg of cefoxitin to a 30-μg cefuroxime disk.

Effect of induction on constitutive β-lactamase producers. The effect of induction was measured with 17 antimicrobial agents and 23 enterobacter strains which produced β-lactamase constitutively. The data in Fig. 3 show that constitutive enterobacters were much more resistant to β-lactam antibiotics than were the inducible strains shown in Fig. 1. The data shown in Fig. 3 for the cefoperazone-sulbactam combination and cefoperazone alone suggest that sulbactam inhibits β-lactamase in constitutive enterobacters. All constitutive organisms were resistant to cefuroxime, cefamandole, cefazolin, cephalothin, cefmetazole, ceftizoxime, and ticarcillin with and without clavulanic acid regardless of induction.

Effect of induction on noninducible, nonconstitutive enterobacters. Organisms with baseline β-lactamase levels of <25 U, induced β-lactamase levels of <750 U, and no inducible resistance to cefuroxime were classified as low producers. Only 3 of 62 enterobacter strains in our collection fit the low-producer category. Addition of cefoxitin to cefuroxime disks failed to decrease the zone sizes of low producers (see Fig. 6).

Baseline β-lactamase levels in inducible, constitutive, and

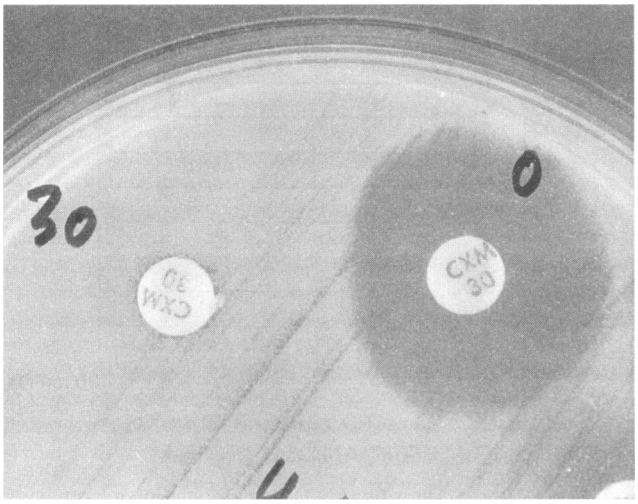

FIG. 2. Typical appearance of cefuroxime susceptibility with and without cefoxitin when testing an enterobacter with inducible β-lactamase. The labels 0 and 30 indicate the amounts (in micrograms) of cefoxitin added to 30-μg cefuroxime (CXM) disks. Note that addition of cefoxitin led to cefuroxime resistance (left) and flattening of the susceptibility zone around the neighboring disk (right).

low-producer enterobacter isolates. β-Lactamase was measured in uninduced enterobacter isolates to determine baseline levels. The distribution curves in Fig. 4 show that high baseline β-lactamase levels occurred more frequently in constitutive organisms. All enterobacter strains with baseline enzyme specific activity below 200 U were susceptible to cefuroxime, and those with activity over 200 U were resistant.

Average β-lactamase activity in induced and uninduced enterobacter groups. β-Lactamase was measured in induced and uninduced enterobacters. Average baseline and induced

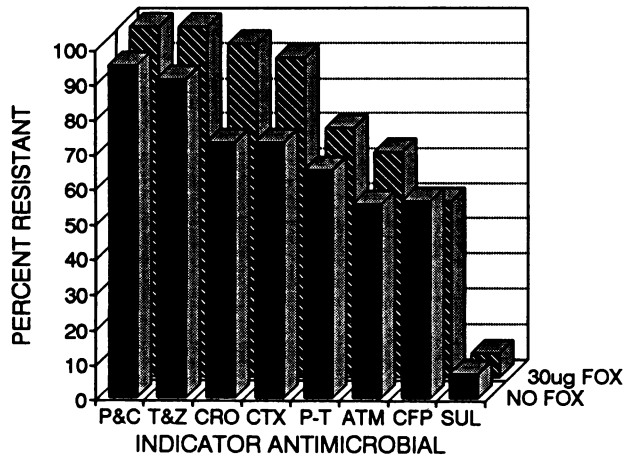

FIG. 3. Effect of cefoxitin (FOX) on the resistance of 23 enterobacters which produce β-lactamase constitutively. Disk diffusion results obtained with the following antimicrobial agents are shown: piperacillin and cefotetan (P&C), ticarcillin and ceftazidime (T&Z), ceftriaxone (CRO), cefotaxime (CTX), piperacillin with tazobactam (P-T), aztreonam (ATM), cefoperazone (CFP), and cefoperazone with sulbactam (SUL). Piperacillin and cefotetan were tested separately, but the results are combined in the P&C bar because they were identical, as were the ticarcillin and ceftazidime results.

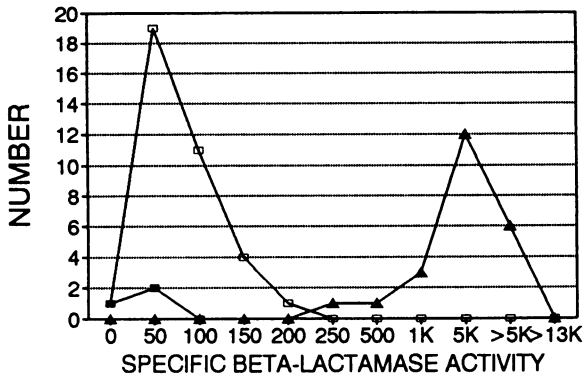

FIG. 4. Distribution of baseline, i.e., uninduced, specific β-lactamase activity in enterobacters which produce low levels despite induction (■; n = 3), are inducible (□; n = 36), or produce β-lactamase constitutively (▲; n = 23). Specific β-lactamase activity is expressed in units of β-lactamase per milligram of protein.

β-lactamase levels for the inducible, constitutive, and low-producer groups are shown in Fig. 5. Increases in β-lactamase occurred with cefoxitin exposure in all groups. Increases in β-lactamase were concomitant with cefuroxime resistance in inducible organisms. β-Lactamase production among low producers was not sufficient for cefuroxime resistance. Constitutive strains produced more β-lactamase when grown with an inducer, but resistance was not a predictable consequence with any antibiotic.

The sources and identities of the isolates studied are listed in Table 1, which summarizes the abilities of individual isolates to respond to cefoxitin induction with (i) β-lactamase production and (ii) the corresponding resistance(s).

Titration of inducer concentration. A range of cefoxitin concentrations was tested to determine the most effective inducer dose. The curves in Fig. 6 show the effect of the cefoxitin concentration on average zone sizes among the three groups of enterobacters tested versus cefuroxime. Organisms

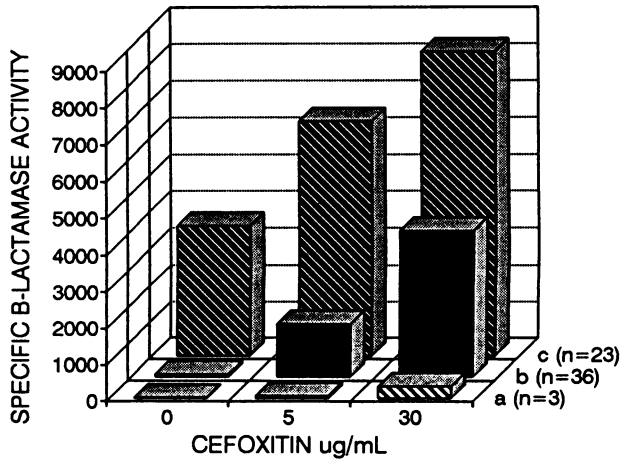

FIG. 5. Effect of induction on the average β-lactamase specific activity in low β-lactamase-producing (a), inducible (b), and constitutive (c) groups of enterobacters. Specific activity ranges: a, low producers (0 to 23, 59, and 0 to 311 U/mg of protein) b, inducible (0 to 199, 329 to 4,017, and 1,370 to 11,750 U/mg of protein) c, constitutive (205 to 12,014, 1,235 to 32,922, and 1,531 to 58,124 U/mg of protein). The organisms were grown in 0, 5, and 30 μg of cefoxitin per ml, respectively.

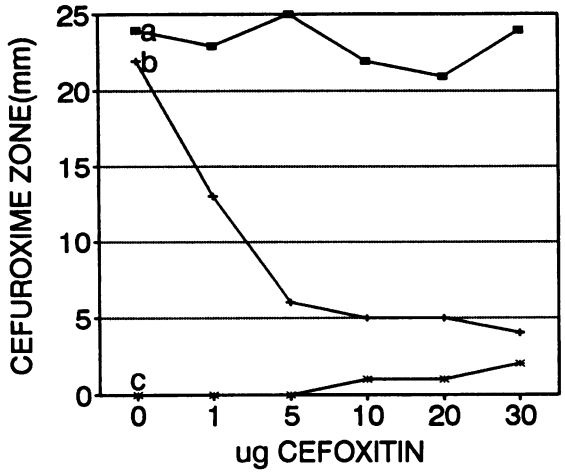

FIG. 6. Effect of cefoxitin on sizes of cefuroxime-produced zone around enterobacters with respect to the β-lactamase production type. a, low producers (n = 3), b, inducible (n = 36), and c, constitutive (n = 23). Cefoxitin was added in 5 μl.

in the inducible group showed a dose-response curve indicating that 30 μg of cefoxitin is optimal for expression of resistance. Testing of inducible strains *E. aerogenes* U24 and *E. cloacae* 55 showed that cefuroxime zones of inhibition did not decrease further with 40, 60, 80, 90, or 120 μg of cefoxitin. The zone of inhibition for *E. cloacae* 55 was 0 with 10 to 40 μg of cefoxitin but increased to 7 mm with 60 μg and to 10 mm with 120 μg.

DISCUSSION

In a recent review, Ehrhardt and Sanders (5) described five genes involved in β-lactamase production and regulation in enterobacters. Two genes, *ampE* and *ampG*, are believed to encode products involved with the induction process, but the exact mechanism is unknown. *ampC* encodes β-lactamase. *ampR* encodes a regulatory protein which enhances *ampC* expression in the presence of an inducing signal. Wild-type enterobacters are inducible, and mutants lacking *ampR* are not inducible. *ampD* encodes a repressor. Mutants lacking *ampD* are stably derepressed enterobacters which produce high levels of β-lactamase constitutively and are probably selected for by many β-lactam antibiotics to which they are resistant. Ehrhardt and Sanders (5) have postulated that the frequency of constitutive resistance may be related to antibiotic selective pressure in a hospital or a community. Enterobacters which produced β-lactamase constitutively constituted only 16% of the Olin E. Teague Veterans' Hospital consecutive isolates but 59% of the St. Louis University Hospital enterobacters collected from serious infections and treatment failures. While a concern, resistance in constitutive organisms is expressed in standard susceptibility tests but wild-type organisms can spuriously appear to be susceptible if an inducer is not present to activate upregulation of *ampC* by the *ampR* gene.

Disk approximation has been the most widely used test for detection of resistance due to inducible β-lactamase. Sanders and Sanders (15) used cefoxitin as an inducer and cefamandole disks as indicators. Thore et al. (19) proposed cefotaxime as the indicator antimicrobial agent. Nadal and von Graevenitz (10) proposed interposing cefoxitin inducer disks with the expanded-spectrum cephalosporins ceftazidime, ceftriaxone, cefotaxime, and latamoxef. Cefamandole detected resistance in 88% of inducible enterobacters as judged by ≥4-mm zone flattening (15). Thore et al. (19) reported that 90% of enterobacters gave ≥1-mm zone truncation with cefotaxime when induced with imipenem or cefoxitin (combined data). Reproducibility was 80 and 81% for imipenem and cefoxitin, respectively. Also using cefoxitin induction and the 1-mm criterion, Nadal and von Graevenitz (10) reported that 88% of enterobacters had inducible resistance to at least one of four expanded-spectrum cephalosporins tested, the best indicator being ceftazidime, which detected resistance in 51%. Problems with disk approximation include the distance between disks, the strength of the inducer, the choice of indicator antimicrobial agent, and the degree of zone truncation needed to correlate with resistance. The test we have described allows interpretation of resistance on the basis of National Committee for Clinical Laboratory Standards criteria for disk diffusion testing (11). Inducible enterobacters were susceptible to cefuroxime in the uninduced state but when induced they usually showed absolutely no zone of inhibition with cefuroxime susceptibility test disks. Cefoxitin-induced cefuroxime resistance correlated perfectly with β-lactamase inducibility in enterobacters.

The use of disk approximation to detect inducible β-lactamases in *Pseudomonas* sp., *Serratia* sp., *Providencia* sp., *Morganella* sp., and *Proteus vulgaris* has been reported (10, 15, 19). Our cefoxitin inducer-cefuroxime indicator method detected resistance in zero of nine *Providencia*, zero of five *Pseudomonas*, one of two *Morganella*, and one of four *Serratia* strains. Resistance in *Serratia* and *Morganella* strains emerged with 1 μg of cefoxitin, but organisms became as susceptible as controls with as little as 5 or 10 μg because, unlike enterobacters, these isolates were susceptible to cefoxitin. All pseudomonads were resistant to cefuroxime prior to induction, obviating its use as an indicator. Perhaps a similar method with a different inducer(s) and/or indicator(s) can be found to measure inducible resistance in non-enterobacter organisms definitively.

Isolates 1088 and U61 initially gave discordant results with respect to β-lactamase production type and cefuroxime susceptibility. The discordant cultures were found to consist of heterogeneous populations which could be separated as follows: *E. aerogenes* 1088, *E. aerogenes* 1088A (low β-lactamase, not inducible, maintained susceptibility to cefuroxime and cefoxitin) and 1088B (constitutive, consistently resistant to cefuroxime and other β-lactam antibiotics); *E. cloacae* U61, *E. cloacae* U61A (inducible, resistant to cefuroxime only when induced) and U61B (constitutive, persistently resistant to cefuroxime and other β-lactams antibiotics). These two examples indicate that mixed populations with respect to β-lactamase production and resistance may be common among enterobacters and that constitutive mutants may arise from highly susceptible weak β-lactamase producers, as well as inducible organisms. Use of the progeny of a single colony for all tests minimized discordant results due to heterogeneous populations that seemed to arise spontaneously.

If reduction of zone size is related to substrate specificity, then it appears that inducible β-lactamase(s) most readily hydrolyzes cefmetazole, cephalothin, cefazolin, cefuroxime, cefamandole, ceftriaxone, cefotaxime, cefotetan, ticarcillin, ceftizoxime, aztreonam, and ceftazidime while cefoperazone and piperacillin are resistant to hydrolysis. Preliminary data obtained with crude lysates agree with the above order, except that hydrolysis of cefuroxime appears to be slower than that of cefamandole.

In the light of the findings of Ehrhardt and Sanders (5), it is not surprising that inducible enzymes were not inhibited by clavulanic acid. This is probably due to poor binding, as reported by Cullman (3). The efficacy of tazobactam and

sulbactam as inhibitors of inducible β-lactamase was difficult to assess because inducible organisms were all susceptible to piperacillin and cefoperazone regardless of induction. More of the constitutive organisms were susceptible when tazobactam was present with piperacillin and when sulbactam was tested with cefoperazone. As a result of induction of constitutive organisms, only one organism acquired resistance to piperacillin-tazobactam. None of 10 constitutive organisms was susceptible to piperacillin, but 6 of 10 uninduced and 5 of 10 induced organisms were susceptible to piperacillin-tazobactam. The average zone sizes for the 10 organisms were 12 mm with piperacillin and 17 mm with piperacillin-tazobactam, regardless of induction. Regardless of induction, none of 12 constitutive organisms was susceptible to cefoperazone but 11 of 12 were susceptible to cefoperazone-sulbactam. The average zone sizes were 15 mm for cefoperazone and 20 mm for cefoperazone-sulbactam. Thus, tazobactam and sulbactam make piperacillin and cefoperazone more effective against constitutive enterobacters, and efficacy is maintained with extremely high β-lactamase levels. Cullman (3) reported that the inducible β-lactamase of enterobacters has a higher binding affinity for tazobactam than sulbactam. Our data seem to indicate the reverse, but that may be due to the efficacy of the companion antimicrobial agent or the combination rather than the affinity of the inhibitor. The clinical efficacy of tazobactam and sulbactam versus the inducible β-lactamase of enterobacters remains to be proven.

The cefoxitin inducer-cefuroxime indicator system works well in unveiling latent inducible β-lactamase. Cefuroxime is an ideal indicator in that induced β-lactamase caused resistance while latent β-lactamase producers were susceptible. Induced resistance to cefuroxime was sometimes, but not always, accompanied by increased resistance to other broad- and expanded-spectrum cephalosporins and ticarcillin in our disk diffusion-induction studies. Inducible β-lactamase was uniformly detected by acquisition of resistance to cefuroxime, and Seeberg et al. (18) and Ehrhardt and Sanders (5) have stated that inducible β-lactamase is responsible for the resistance of enterobacters to expanded-spectrum cephalosporins. We demonstrated the ability of crude lysates from induced organisms to degrade ceftriaxone, cefotaxime, and ceftazidime. Our cefoxitin inducer-cefuroxime indicator system is simple enough to be employed in routine laboratory practice to reliably identify organisms capable of producing inducible β-lactamase and its implied resistance.

ACKNOWLEDGMENTS

We thank Thomas W. Milligan for sending us *Enterobacter* isolates from St. Louis University Hospital and for advice and encouragement. We acknowledge the excellent technical assistance of Mollie Huber and Samuel Huber in developmental work which led to this report and Laura Huber for technical assistance for the work presented here. We also gratefully acknowledge Dennis Jay for consultation on automated assay development.

REFERENCES

1. **Barry, A. L., R. N. Jones, and the Collaborative Antimicrobial Susceptibility Testing Group.** 1988. Criteria for disk susceptibility tests and quality control guidelines for the cefoperazone-sulbactam combination. J. Clin. Microbiol. **26:**13–17.
2. **Beckwith, D. G., and J. A. Jahre.** 1980. Role of a cefoxitin-inducible beta-lactamase in a case of breakthrough bacteremia. J. Clin. Microbiol. **12:**517–520.
3. **Cullman, W.** 1990. Interaction of beta-lactamase inhibitors with various beta-lactamases. Chemotherapy **36:**200–208.
4. **Cullman, W., A. Dalhoff, and W. Dick.** 1984. Nonspecific induction of beta-lactamase in *Enterobacter cloacae*. J. Gen. Microbiol. **130:**1781–1786.
5. **Ehrhardt, A. F., and C. C. Sanders.** 1993. Beta-lactamase resistance amongst *Enterobacter* species. J. Antimicrob. Chemother. **32**(Suppl. B):1–11.
6. **Gatus, B. J., S. M. Bell, and A. S. Jimenez.** 1988. Comparison of glycine enhancement with cefoxitin induction of class I beta-lactamase production in *Enterobacter cloacae* ATCC 13047. J. Antimicrob. Chemother. **21:**163–170.
7. **Jones, R. N., H. W. Wilson, W. J. Novick, Jr., A. L. Barry, and C. Thornsberry.** 1982. In vitro evaluation of CENTA, a new beta-lactamase-susceptible chromogenic cephalosporin reagent. J. Clin. Microbiol. **15:**954–958.
8. **Lindh, E., K. Dornbusch, K. Jalakas, and A. Forsgren.** 1990. Antibiotic susceptibility and beta-lactamase production in clinical isolates of *Enterobacter* spp. APMIS **98:**462–470.
9. **Livermore, D. M., M. Akova, P. Wu, and Y. Yang.** 1989. Clavulanate and beta-lactamase induction. J. Antimicrob. Chemother. **24**(Suppl. B):23–33.
10. **Nadal, D., and A. von Graevenitz.** 1993. Routine detection of inducible beta-lactamase in gram-negative bacilli by means of disk susceptibility testing. J. Microbiol. Methods **17:**283–291.
11. **National Committee for Clinical Laboratory Standards.** 1993. Performance standards for antimicrobial disk susceptibility tests, 5th ed. Approved standard M2-A5. National Committee for Clinical Laboratory Standards, Villanova, Pa.
12. **Okonogi, K., A. Sugiura, M. Kuno, E. Higashide, M. Kondo, and A. Imada.** 1985. Effect of beta-lactamase induction on susceptibility to cephalosporins in *Enterobacter cloacae* and *Serratia marcescens*. J. Antimicrob. Chemother. **16:**31–42.
13. **Preheim, L. C., R. G. Penn, C. C. Sanders, R. V. Goering, and D. K. Giger.** 1982. Emergence of resistance to beta-lactam and aminoglycoside antibiotics during moxalactam therapy of *Pseudomonas aeruginosa* infections. Antimicrob. Agents Chemother. **22:** 1037–1041.
14. **Sanders, C. C.** 1984. Failure to detect resistance in antimicrobial susceptibility tests. A "very major" error of increasing concern. Antimicrob. Newsl. **1:**24–34.
15. **Sanders, C. C., and W. E. Sanders, Jr.** 1979. Emergence of resistance to cefamandole: possible role of cefoxitin-inducible beta-lactamases. Antimicrob. Agents Chemother. **15:**792–797.
16. **Sanders, C. C., and W. E. Sanders, Jr.** 1983. Emergence of resistance during therapy with the newer beta-lactam antibiotics: role of inducible beta-lactamases and implications for the future. Rev. Infect. Dis. **5:**639–648.
17. **Sanders, C. C., and W. E. Sanders, Jr.** 1985. Microbial resistance to newer generation beta-lactam antibiotics: clinical and laboratory implications. J. Infect. Dis. **151:**399–406.
18. **Seeberg, A. H., R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1983. Chromosomal β-lactamases of *Enterobacter cloacae* are responsible for resistance to third-generation cephalosporins. Antimicrob. Agents Chemother. **23:**918–925.
19. **Thore, M., K. Jalakas, I. Eriksson, and K. Dornbusch.** 1989. Evaluation of a disk approximation test of inducible beta-lactamases in *Enterobacteriaceae* and *Pseudomonas aeruginosa*. APMIS **97:**317–324.