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Use of Several Inducer and Substrate Antibiotic Combinations in a Disk Approximation Assay Format To Screen for AmpC Induction in Patient Isolates of *Pseudomonas aeruginosa*, *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp.

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Two-hundred consecutive, single patient isolates of *Enterobacter* spp., *Serratia* spp., *Citrobacter* spp., and *Pseudomonas aeruginosa* were evaluated for AmpC production using a variety of inducer-substrate antibiotic combinations in a disk approximation format. The combinations examined included ceftazidime-piperacillin, imipenem-ceftazidime, imipenem-ceftazidime, imipenem-piperacillin-tazobactam, and imipenem-ceftazidime. All isolates were also screened for the presence of extended-spectrum β -lactamase (ESBL) activity. In total, 85.5% of isolates were shown to be inducible for the production of AmpC by one or more inducer/substrate combinations and 11% of all isolates were stably derepressed for the expression of AmpC. Of all of the combinations, imipenem/piperacillin-tazobactam provided the greatest sensitivity (97.1%). All combinations were 100% specific when a positive test was observed. Given this background among these organisms in our institution, it is reasonable to develop an antibiotic reporting strategy that favors the selection of agents for therapy of these organisms that do not serve as labile substrates of AmpC.

Antimicrobial therapy of serious infections caused by *Enterobacter* and *Serratia* species, *Citrobacter freundii*, and *Pseudomonas aeruginosa* (*ESCP*) can be problematic since many of these organisms possess inducible AmpC β -lactamases (AmpC) that can be upregulated by subinhibitory concentrations of certain β -lactam antibiotics (6, 11, 14). Further, mutations can occur in the regulatory components of AmpC leading to a stable, hyperproduction of AmpC with concomitant high-level resistance to many classes of β -lactam antibiotics (14). The ability of various β -lactam antibiotics to induce AmpC synthesis in *ESCP* organisms varies with some agents such as ampicillin, ceftazidime, and carbapenems acting as strong inducers. Extended-spectrum cephalosporins such as ceftazidime and cefotaxime, and ureidopenicillins do not readily induce but can serve as substrates of AmpC (11).

Routine antimicrobial susceptibility testing using either disk diffusion or broth microdilution can generally differentiate between strains of *ESCP* whose AmpC enzymes remain uninduced or are hyperproduced based on resistance profiles versus a spectrum of β -lactam antibiotics. However, these methods cannot readily predict which strains carry inducible AmpC enzymes. In the absence of this information, reporting algorithms could be established wherein resistance to all narrow-, expanded-, and broad-spectrum cephalosporins, ureidopenicillins, and carboxypenicillins (the latter two with or without β -lactamase inhibitors) is assumed considering the potential for selection of stably derepressed or induced AmpC-producing strains during therapy. More practically, however, it would be useful to de-

velop a simple screening assay to detect inducible AmpC that would have sufficient sensitivity and specificity for *ESCP* to allow reporting along with susceptibility results. The purpose of the present study was twofold. The first was to evaluate the performance of several inducer/substrate combinations in order to establish the AmpC status of *ESCP*. The second was to screen a representative number of *ESCP* isolates from our institution to determine whether a reporting algorithm relative to β -lactam antibiotic testing was a reasonable alternative to AmpC screening.

MATERIALS AND METHODS

Bacterial strains. For the present study, 200 consecutive single patient isolates of *Enterobacter*, *Serratia*, and *Citrobacter* spp. and *P. aeruginosa* were collected over a 3-month period from May through July 2005. Isolates were identified by using either the VITEK2 colorimetric or API identification systems (bioMérieux SA, Marcy l'Etoile, France). The collection consisted of 134 *P. aeruginosa* strains and 13 *Citrobacter* (9 *freundii*, 3 *koserii*, and 1 *amalonaticus*), 13 *Serratia marcescens*, and 40 *Enterobacter* (24 *cloacae* and 16 *aerogenes*) isolates. All isolates were either tested immediately or maintained on Trypticase soy agar slants at room temperature for up to 48 h prior to testing.

Induction of AmpC. The induction of AmpC synthesis was based on the disk approximation (D-test) assay as previously described (9) using ceftazidime/piperacillin (FOX/PIP) and adding several other inducer/substrate combinations for comparison, including imipenem/cefotaxime (IMI/CTX), imipenem/cefazidime (IMI/CAZ), imipenem/piperacillin-tazobactam (IMI/TZP), and imipenem/ceftazidime (IMI/FOX). In addition, comparisons were made between zone sizes produced by a disk containing CAZ alone and one with ceftazidime and clavulanate (CAZ-CLAV) to look for evidence of extended-spectrum β -lactamase (ESBL) production and/or AmpC induction by clavulanate. Inhibitory zone sizes generated by a PIP disk and a TZP disk were compared to detect tazobactam induction of AmpC. All disks were obtained from BBL (Becton Dickinson, Sparks, MD) at the following potencies: TZP (100 and 10 μ g, respectively [100 μ g-10 μ g]), PIP (100 μ g), IMI (10 μ g), CAZ-CLAV (30 and 10 μ g, respectively), CAZ (30 μ g), FOX (30 μ g), and CTX (30 μ g).

To perform the assay, disk diffusion susceptibility testing was performed according to the CLSI guidelines (15). Inducer/substrate disks were placed on the surface of

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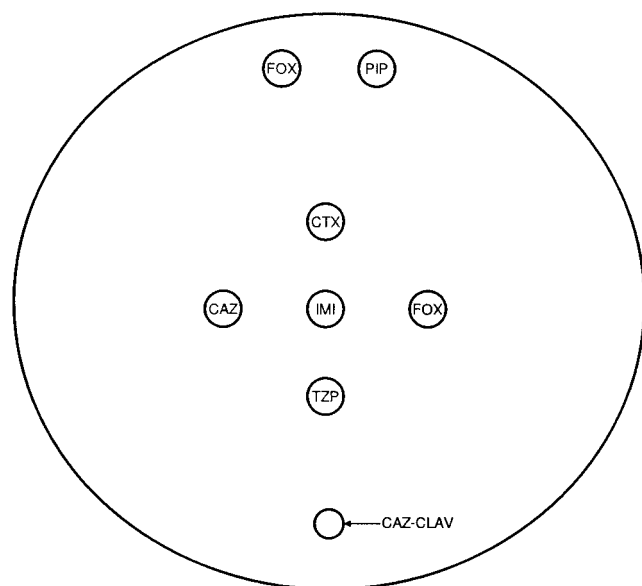


FIG. 1. Template used for the placement of antibiotic disks on the surface of a 140-mm Mueller-Hinton agar plate (BBL) to test for the induction of AmpC. Inducer/substrate combination disks were placed adjacent to one another at a distance of 25 mm on center. Disk potency is as follows: TZP (100 μ g-10 μ g), PIP (100 μ g), IMI (10 μ g), CAZ-CLAV (30 μ g-10 μ g), CAZ (30 μ g), FOX (30 μ g), and CTX (30 μ g).

Mueller-Hinton agar plates (BBL) at a distance of 25 mm on center using a template (Fig. 1). After incubation, zones of inhibition were measured on both the induced (adjacent to the inducer disk) and the uninduced side of the substrate disk from disk edge to zone edge. A test was considered positive if the zone of inhibition was reduced by ≥ 2 mm on the induced side of the substrate disk.

Quality control of the performance of the assay was accomplished with positive (*P. aeruginosa* ATCC 27853) and negative (*Escherichia coli* 25922) control strains. Assays performed on 5 consecutive days provided positive results for each inducer/substrate pair except IMI/FOX with the positive control. Negative results were obtained for each inducer/substrate combination with the *E. coli* ATCC 25922 except the IMI/FOX pair where a faint blunting of the induced side of the FOX disk was observed. It has previously been noted that this strain produces low levels of AmpC that might contribute to this observation (3). Neither control strain showed any zone size difference between the CAZ-CLAV and CAZ or the PIP and TZP disks.

Study strains were considered to be noninducible if none of the substrate/inducer combinations produced a positive test and the isolate was not stably derepressed for AmpC. Strains were considered inducible if a positive test was obtained with any of the inducer/substrate combinations or if the zone of inhibition produced by the CAZ-CLAV disk was ≥ 2 mm less than the zone produced by a CAZ disk. Strains were considered to be stably derepressed if resistance was observed to all substrate drugs and could be confirmed by inhibition of AmpC activity without evidence of metallo- β -lactamase production. The induction or

derepression of AmpC for all strains was confirmed with a disk potentiation test (18) using 3-aminophenylboronic acid (APB; Sigma-Aldrich, St. Louis, MO) as an inhibitor of AmpC β -lactamases. Strains identified as resistant to IMI were also evaluated for metallo- β -lactamase production using the double disk synergy method of Arakawa et al. (2) with 2-mercaptopyruvic acid as a specific inhibitor of enzyme activity.

RESULTS

***P. aeruginosa*.** A total of 134 strains of *P. aeruginosa* were collected among the 200 total isolates included in the study. Of these, 115 (85.8%) were shown to be inducible for AmpC by one or more of the substrate/inducer combinations by using an affirmative confirmation test. The sensitivity of each screening combination was as follows: FOX/PIP, 40%; IMI/CTX, 69%; IMI/CAZ, 100%; and IMI/TZP, 100% (Table 1). The combination of IMI/FOX was not useful for evaluating strains of *P. aeruginosa* since none of the isolates produced a zone of inhibition surrounding the FOX disk. A comparison of the zone sizes produced by CAZ and CAZ-CLAV was useful for the detection of AmpC induction in seven strains (6.2%) and six of these seven isolates were also resistant to IMI. Tazobactam induced AmpC in three strains as the zone size generated by TZP was ≥ 2 mm smaller than that produced by PIP alone. The specificity of all substrate/inducer combinations was determined to be 100% by the APB confirmation assay. The mean difference in inhibitory zone size between the induced and uninduced sides of the substrate disk gave the following rank for the AmpC induction test: IMI/CTX > IMI/TZP > IMI/CAZ > FOX/PIP (Table 1).

Of 134 isolates of *P. aeruginosa*, 15 (11.2%) were stably derepressed for the production of AmpC. Of these, six were also resistant to IMI, which was reversed by ABP. None of the IMI-resistant strains were shown to produce a metallo- β -lactamase using zone expansion with 2-mercaptopyruvic acid.

Only 4 of 134 strains of *P. aeruginosa* showed no induction of AmpC using any of the substrate/inducer combinations. All of these were susceptible to IMI, CTX, CAZ, PIP, and TZP, and none demonstrated an enhanced zone of inhibition with the addition of APB (data not shown).

***Enterobacter* spp.** A total of 40 *Enterobacter* spp. (24 *E. cloacae* and 16 *E. aerogenes*) were collected during the study period. Twenty-two of twenty-four strains of *E. cloacae* and twelve of sixteen *E. aerogenes* isolates (90% of the total) were shown to be inducible for AmpC synthesis by one or more of the inducer/substrate combinations (Table 1). Of the 40 isolates, 6 (4 *E. aerogenes* and 2 *E. cloacae*) were stably derepressed for Amp C

TABLE 1. Sensitivity of AmpC induction screening assay for each inducer/substrate combination examined and mean difference in inhibitory zone size between the uninduced and induced side of the substrate disk for a positive test (≥ 2 mm)^a

Organism	% Sensitivity, mean difference (mm) ^a				
	FOX/PIP	IMI/CTX	IMI/CAZ	IMI/TZP	IMI/FOX
<i>Pseudomonas aeruginosa</i>	40, 2.4 (2)	69, 5.2 (6)	100, 3.7 (5)	100, 4.4 (7)	0, NA ^b (NA)
<i>Enterobacter</i> spp.	29.4, 2.4 (2)	85.2, 3.9 (6)	76.4, 3.4 (4)	94.1, 2.9 (3)	2.9, 4.0 (4)
<i>Citrobacter</i> spp.	30, 2.0 (0)	90, 4.3 (4)	90, 2.8 (2)	80, 2.9 (2)	30, 4.5 (2)
<i>Serratia</i> spp.	66.7, 3.3 (4)	100, 3.3 (3)	91.7, 2.4 (3)	91.7, 3.4 (5)	8.3, 6 (4)
All organisms	39.8, 2.5 (4)	75.4, 4.7 (6)	94.2, 3.5 (5)	97.1, 4.0 (7)	2.3, 3.8 (4)

^a The absolute range (maximum difference minus 2 mm) is shown in parentheses. The specificity of each screening combination was 100%.

^b NA, not applicable.

production, and none were noninducible. AmpC expression was confirmed for all inducible and derepressed strains using APB. The sensitivity of each screening combination for inducible *Enterobacter* species was as follows: FOX/PIP, 29.4%; IMI/CTX, 85.2%; IMI/CAZ, 76.4%; and IMI/TZP, 94.1% (Table 1). As with *P. aeruginosa*, the combination of IMI/FOX was not useful (sensitivity = 2.9%) because most strains were resistant to FOX. The rank order of screening combinations for *Enterobacter* species in terms of zone reduction was as follows: IMI/CTX > IMI/CAZ > IMI/TZP > FOX/PIP for a positive induction test (Table 1).

Five isolates of *E. cloacae* were shown to produce both inducible AmpC and ESBL activity as determined by comparison of the CAZ-CLAV and CAZ disks. This finding was confirmed according to the CLSI protocol (15, 16). The only screening test able to detect inducible AmpC in these five isolates was the IMI/TZP combination. Six *Enterobacter* isolates (4 *E. aerogenes* and 2 *E. cloacae*) were stably derepressed for the production of AmpC and confirmed by APB inhibition. None of the 40 *Enterobacter* strains was resistant to IMI, produced a metallo- β -lactamase, or was noninducible. Comparison of the PIP and TZP zone sizes gave no indication of tazobactam induction of AmpC in *Enterobacter* species.

Citrobacter spp. Thirteen *Citrobacter* spp. consisting of 9 *C. freundii*, 3 *C. koseri*, and 1 *C. amalonaticus* isolate were among the study isolates. AmpC induction was demonstrable for 10 of these (8 *C. freundii* and 1 each *C. koseri* and *C. amalonaticus*) with one or more inducer/substrate combinations. One isolate of *C. freundii* was stably derepressed for AmpC synthesis, and two isolates of *C. koseri* could not be induced. AmpC activity was confirmed for all inducible and derepressed strains with APB, and none of the isolates was resistant to IMI or had evidence of metallo- β -lactamase activity. The sensitivities of each inducer/substrate combinations to detect AmpC induction were as follows: FOX/PIP, 30%; IMI/CTX, 90%; IMI/CAZ, 90%; IMI/TZP, 80%; and IMI/FOX, 30% (Table 1). One strain of *C. freundii* demonstrated AmpC induction by comparison of the CAZ-CLAV and CAZ disks but was also positive with three other screening combinations. None of the *Citrobacter* isolates showed tazobactam induction of AmpC. For *Citrobacter* species, the rank order of zone reduction was as follows: IMI/CTX > IMI/FOX > IMI/TZP > IMI/CAZ > FOX/PIP (Table 1).

Serratia spp. All 13 *Serratia* species collected for the study were identified as *S. marcescens*, and 12 of 13 gave a positive induction test with one or more of the inducer/substrate combinations. The sensitivity of each combination for the detection of AmpC induction with *S. marcescens* was FOX/PIP, 66.7%; IMI/CTX, 100%; IMI/CAZ, 91.7%; IMI/TZP, 91.7%; and IMI/FOX, 8.3% (Table 1). AmpC induction by clavulanic acid was observed in 2 of the 12 isolates by comparing the CAZ-CLAV and CAZ zone sizes, but both were identified by three or more of the inducer/substrate combinations. None of the isolates showed evidence of ESBL or metallo- β -lactamase production, but one strain appeared to be induced for AmpC production in the presence of tazobactam. None of the isolates was stably derepressed for AmpC synthesis, and one could not be induced with any of the combinations tested. For *Serratia* species, the rank order of zone reduction for individual screens was as follows: IMI/TZP > IMI/CTX > FOX/PIP > IMI/

CAZ. The combination of IMI/FOX was only positive for one isolate (6-mm difference).

Cumulative results. A total of 171 of the 200 *ESCP* isolates collected for the present study (85.5%) could be induced for synthesis of AmpC based on a positive test with at least one of the screening combinations evaluated and confirmed with the addition of APB. The sensitivities of the various screening combinations for all *ESCP* isolates were as follows: IMI/FOX, 2.3%; FOX/PIP, 39.8%; IMI/CTX, 75.4%; IMI/CAZ, 94.2%; and IMI/TZP, 97.1%. The comparison of zones produced by CAZ-CLAV and CAZ led to the detection of AmpC induction in 11 isolates (6.4%), but all were determined to be positive by two or more of the other screening assays. Tazobactam apparently caused induction of AmpC production in 4 of the 171 isolates (2.3%) but, again, each of these produced a positive screening test with at least two of the other combinations. A total of 22 isolates (11%) were stably derepressed for AmpC production, whereas only 6 of 200 strains (3%) could not be induced for AmpC production with any of the inducer/substrate combinations, including 4 of 134 (3%) *P. aeruginosa*, 2 of 13 *Citrobacter* spp. (15.4%, both *C. koseri*), 1 of 13 *S. marcescens* (7.6%), and no *Enterobacter* species.

DISCUSSION

The purpose of this investigation was twofold: first, to examine the status of AmpC production among *ESCP* from our facility so as to better understand treatment options in the setting of serious systemic infection and, second, to develop a simple and sensitive screening assay to evaluate AmpC activity in these organisms. Using several inducer/substrate combinations, we found that 85.5% of single patient *ESCP* isolates collected for the present study were capable induced synthesis of an AmpC. This finding is not unexpected since it is well known that these genera harbor inducible AmpC enzymes (6, 11, 14). One unusual finding was the identification of one strain each of *Citrobacter koseri* and *C. amalonaticus* that were positive for inducible AmpC. Although AmpC activity has been associated with *C. koseri* (7), we are unaware of reports detailing AmpC activity in *C. amalonaticus*. More importantly, however, this finding serves to remind that inducible AmpC is not monopolized by *C. freundii* but can be shared by other *Citrobacter* species.

A total of 22 isolates (11%) were stably derepressed for AmpC production, including 15 of 134 *P. aeruginosa*, 4 of 40 *Enterobacter* sp., and 1 of 13 *C. freundii* isolates, but no isolates of *S. marcescens*. With respect to *Enterobacter* and *Citrobacter* spp., the frequency of strains demonstrating stable derepression of AmpC is much less than that the 38% reported by Pfaller et al. (17) for the same genera when they examined bloodstream isolates for the SCOPE surveillance program. Our numbers, however, are much smaller and reflect organisms recovered from many sources other than blood.

As a sidelight, we identified 16 isolates of IMI-resistant *P. aeruginosa*. We found that 9 of these demonstrated inducible AmpC production, while 7 isolates were stably derepressed for AmpC production. IMI activity was potentiated in all isolates by ABP but not with 2-mercaptopropionic acid, indicating that IMI resistance was mediated by a combination of AmpC and

loss of OprD (D2 porin protein) as previously described (9, 10, 12, 19) and not through acquisition of a metallo- β -lactamase.

The choice of inducer/substrate combinations evaluated in the present study was limited to candidates currently part of our susceptibility panel. Only two inducers were considered (IMI and FOX) based on previous observations that the former is a strong inducer but a poor substrate for AmpC activity, while the latter is both a strong inducer and a labile substrate (11). Likewise, substrates were selected for known susceptibility to hydrolysis by AmpC enzymes. One combination (FOX/PIP) was chosen based on a previous study demonstrating 100% sensitivity for the detection of inducible AmpC among 10 clinical isolates of *P. aeruginosa* (9). Our study found poor sensitivity ($\leq 40\%$) of this combination for the detection of inducible AmpC in *P. aeruginosa*, *Enterobacter* spp., and *Citrobacter* spp. and for all ESCP organisms combined. As a substrate, FOX fared less well when combined with IMI as an inducer where the combination had an overall sensitivity of 2.3% for ESCP. The poor performance of this combination can be explained by the high rate of resistance of study isolates to FOX. A model substrate for this assay requires a zone of inhibition of ≥ 2 mm on the uninduced side of the disk to register a positive response on the induced side. In the case of FOX/IMI, only 23 of 200 test organisms generated a zone of inhibition of >2 mm on the uninduced side of the disk, and most had no zone at all. Although the sensitivity of the IMI/CTX combination was 100% for *Serratia* species, the overall sensitivity was only 75.4% for all isolates—a result of resistance among *P. aeruginosa* (50 of 134 strains with a zone of inhibition of <2 mm on the uninduced side of the CTX disk). We found that the combination of IMI/CAZ or IMI/TZP provided excellent sensitivity (94.2 and 97.1%, respectively) for the detection of inducible AmpC. The greater sensitivity of the latter is partially due to background ESBL activity. All five *E. cloacae* isolates with ESBL expression had a positive test for induced AmpC using the IMI/TZP combination and a negative result with the IMI/CAZ pair. The mean differences in the inhibitory zone size between the induced and uninduced sides of the substrate disk for the IMI/CAZ and IMI/TZP combinations were 3.5 and 4.0 mm, respectively, so that either test was easy to interpret. Regardless of the substrate/inducer combination, the specificity of a positive test was determined to be 100% based on ABP inhibition of AmpC activity.

It can be argued that the selection of an antimicrobial agent for infections caused by ESCP organisms should be based on the assumption that certain drugs such as FOX or extended-spectrum cephalosporins might increase the risk of selecting stably derepressed AmpC-producing strains during therapy (4, 5, 11, 13). Other agents, such as piperacillin, seem to be less likely to select for derepressed mutants in vitro but clearly have reduced activity against strains already derepressed for AmpC production (11). If a simple screening test for AmpC induction was available, it would be possible to modify antibiotic reporting cascades so as to avoid utilization of agents for ESCP that act as substrates of AmpC when dealing with a site of infection where extremely high levels of antibiotics cannot be assured (13). Prior to this analysis, the microbiology laboratory in conjunction with the infectious diseases service and pharmacy had elected to issue a default interpretation of “resistant” for narrow-, extended-, and broad-spectrum cephalosporins and urei-

dopenicillins (including TZP) for *Enterobacter* and *Serratia* spp. and *C. freundii* (actual results were retained and made available by request to infectious diseases and pharmacy personnel). Actual IMI and cefepime results were provided for all ESCP due to the stability of these agents against AmpC (8, 11), although IMI is a restricted agent at our institution and requires approval for use. CAZ is tested against all isolates of *P. aeruginosa*, but the results are suppressed since this is currently not a formulary drug and requires prior approval.

In light of our results, routine screening of ESCP for AmpC induction seems fruitless at our institution given that 85% of isolates are inducible and 11% have stable high-level expression. One exception might include the testing of *Citrobacter* spp. other than *C. freundii*, where inducible AmpC does not strictly align with species. Our current practice of reporting susceptibility results for AmpC-stable β -lactam and carbapenem agents along with other AmpC-independent classes of antibiotics for ESCP, while providing a default resistant interpretation for AmpC-labile agents, seems reasonable given these results. One could argue that TZP is an orphan in this algorithm in that the combination is a poor inducer of AmpC and is potentially useful for treatment of infections caused by ESCP (1, 11). However, TZP proved to be the most sensitive AmpC substrate in the present study when combined with IMI as an inducer. Although tazobactam has not been previously recognized as an inducer of AmpC activity (1), we observed three isolates of *P. aeruginosa* and one of *S. marcescens* that suggested tazobactam induction of AmpC. These results were reproduced in repeat trials and are worthy of additional investigation. Indeed, actual TZP results are reported in our algorithm for *P. aeruginosa*, but its use, like IMI, is restricted and requires approval.

Despite our attempts to utilize single patient isolates for the present study, it is likely that many of the strains represent clonal hospital flora and either over- or underestimate the performance of the screening assays. Nonetheless, these isolates are representative of the mix and proportion of ESCP organisms encountered in our facility. Therefore, it would be reasonable for laboratories considering the use of an AmpC induction screening assay to assess site-specific performance characteristics with local flora.

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