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Evaluation of β-Lactamase Inhibitors in Disk Tests for Detection of Plasmid-Mediated AmpC β-Lactamases in Well-Characterized Clinical Strains of *Klebsiella* spp.

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The diagnostic utility of the AmpC β-lactamase inhibitors LN-2-128, 48-1220, and Syn 2190 in combination with cefotetan (CTT) or cefoxitin in a disk test for the detection of clinical isolates of *Klebsiella* spp. producing plasmid-mediated AmpC β-lactamases (pAmpCs) was evaluated. The combination of Syn 2190 and CTT had a sensitivity of 91%, a specificity of 100%, and a reproducibility of 100% and showed the best potential of using an inhibitor for detection of *Klebsiella* spp. producing pAmpCs.

Many nosocomial isolates of *Klebsiella* spp. producing plasmid-mediated AmpC β-lactamases have been involved in several worldwide outbreaks of infection (3, 13, 20). Often, genes encoding plasmid-mediated AmpC β-lactamases coexist on the same plasmid with genes encoding mechanisms of resistance to other classes of antibiotics, leaving clinicians with limited therapeutic options. Plasmid-mediated AmpC β-lactamases produced by isolates of *Klebsiella pneumoniae* associated with decreased outer membrane permeability can even confer resistance to the carbapenems (3, 5, 12). There are also concerns that treatment failures will occur with certain cephalosporins due to incorrect susceptibility tests when organisms producing plasmid-mediated AmpC β-lactamases appear falsely susceptible. Therapeutic failures with cefotaxime and ceftazidime have occurred with isolates that were susceptible to these drugs in vitro (19). The detection of organisms producing plasmid-mediated AmpC β-lactamases is thus important for infection control purposes and for ensuring effective therapeutic options.

The detection of *Klebsiella* spp. producing plasmid-mediated AmpC β-lactamases has been a difficult task for clinical laboratories. *Klebsiella* spp. lack a chromosomal AmpC β-lactamase. Therefore, nonsusceptibility to one of the cephamycins suggests the presence of an AmpC β-lactamase (10). However, this resistance phenotype in *K. pneumoniae* isolates can also be due to decreased outer membrane permeability (8). For the accurate detection of *Klebsiella* spp. producing plasmid-mediated AmpC β-lactamases, the clinical microbiology laboratory must first detect isolates that are nonsusceptible to the cephamycins and then distinguish between plasmid-mediated AmpC β-lactamase producers and those with decreased outer membrane permeability.

Some of the current detection methods for *Klebsiella* spp. producing plasmid-mediated AmpC β-lactamases such as the three-dimensional test or cefoxitin agar method are technically demanding and time-consuming and, therefore, unsuitable for clinical laboratories to perform on a routine basis (6, 7, 11, 14). Other detection methods such as the Hodge test (previously known as the cloverleaf test [9]) and double-disk test are easier to perform, but results can be difficult to interpret (1, 23). These limitations have precluded their widespread adoption in clinical laboratories. Multiplex PCR for detection of plasmid-meditated AmpC β-lactamases is available as a research tool but is not yet available for routine use in clinical laboratories (21). It would be beneficial if clinical laboratories were able to detect organisms producing plasmid-mediated AmpC β-lactamases with a method that is both simple and inexpensive. Currently, there are no recommendations available from the Clinical and Laboratory Standards Institute (CLSI) (previously known as the National Committee for Clinical Laboratory Standards or NCCLS) for detection of organisms producing plasmid-mediated AmpC β-lactamases (16).

The CLSI has established guidelines for detection of extended-spectrum β-lactamases (ESBLs) in *Escherichia coli* and *Klebsiella* spp., and in 2005 *Proteus* spp. were added. The CLSI ESBL phenotypic confirmation disk test involves testing both cefotaxime and ceftazidime alone and in combination with clavulanic acid. A ≥5-mm increase in zone diameter of cefotaxime and/or ceftazidime in the presence of clavulanic acid compared to when the antibiotic is tested alone is a positive test for an ESBL (16).

Previous studies evaluated the diagnostic utility of the AmpC β-lactamase inhibitors LN-2-128 (4) and 48-1220 (Basilea Pharmaceutical, Basel, Switzerland) (22) for the detection of strains producing plasmid-mediated AmpC β-lactamases in a disk test similar to the CLSI disk test for ESBL confirmation (2). Using 20 well-characterized positive and negative control strains, the inhibitor-based test showed the potential for the detection of organisms producing plasmid-mediated AmpC β-lactamases of cefotetan in combination with LN-2-128 and 48-1220. LN-2-128 and 48-1220 are inhibitors of

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TABLE 1. Results of tests with strains producing plasmid-mediated AmpC β-lactamases

Strain	Organism	Resistance mechanism[b]	Result[a] for:											
			CL1	CL2	FL1	FL2	CR1	CR2	FR1	FR2	CS1	CS2	FS1	FS2
01HNH5	*K. pneumoniae*	ACT-like	P	P	P	P	P	P	P	P	P	P	N	N
01WHS18	*K. pneumoniae*	FOX-/SHV ESBL	P	N	P	P	P	N	P	P	P	P	P	P
01WHS51	*K. pneumoniae*	FOX-like	P	P	P	P	P	N	P	P	P	P	P	P
01WHS4	*K. pneumoniae*	FOX-like/SHV ESBL	P	P	N	N	N	P	N	N	P	P	N	N
01BH5	*K. pneumoniae*	FOX-like	P	N	N	N	P	N	N	N	P	P	P	P
01BH49	*K. pneumoniae*	FOX-5	P	N	N	N	P	N	N	N	P	P	P	P
01IFH13	*K. pneumoniae*	FOX-5	N	N	N	N	N	N	N	N	P	P	N	N
01IFH82	*K. pneumoniae*	FOX-like	P	N	N	N	N	N	N	N	P	P	P	N
01IFH95	*K. pneumoniae*	FOX-like	P	N	P	N	P	N	P	N	P	P	P	P
01JMH89	*K. pneumoniae*	FOX-5/SHV ESBL	N	N	N	N	N	N	N	N	P	P	P	P
01JMH44	*K. pneumoniae*	FOX-like/SHV ESBL	N	P	N	N	N	N	N	N	P	P	P	N
01JMH71	*K. pneumoniae*	FOX-5	P	N	N	N	P	N	N	N	P	P	P	P
01JMH164	*K. pneumoniae*	FOX-5/SHV ESBL	N	N	N	N	N	N	N	N	P	P	N	N
01VCH55	*K. pneumoniae*	FOX-like/SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01AML11	*K. pneumoniae*	FOX-5/SHV,TEM ESBL	P	P	N	P	P	P	N	P	P	P	N	N
01CMH13	*K. pneumoniae*	FOX-5	P	N	N	N	P	N	N	N	P	P	N	P
01CLH42	*K. pneumoniae*	FOX-like	P	P	N	N	P	P	N	N	P	P	P	P
01DVA50	*K. pneumoniae*	FOX-like/SHV ESBL	P	P	N	N	P	P	N	N	P	P	N	N
01DVA56	*K. pneumoniae*	FOX-like	N	N	N	N	N	N	N	N	P	P	N	P
01DVA74	*K. pneumoniae*	FOX-like/SHV ESBL	P	P	N	N	P	N	N	N	P	P	P	P
01DVA78	*K. pneumoniae*	FOX-like/SHV ESBL	P	P	N	N	P	N	N	N	P	P	N	N
01DVA86	*K. pneumoniae*	FOX-like/SHV ESBL	P	N	N	N	P	N	N	N	N	N	N	N
01DVA21	*K. pneumoniae*	FOX-like/SHV ESBL	P	P	N	N	P	P	N	N	P	P	N	N
01DVA28	*K. pneumoniae*	FOX-like/SHV ESBL	P	P	N	N	P	P	N	N	P	P	N	N
01DVA41	*K. pneumoniae*	FOX-like/SHV ESBL	P	P	N	N	P	P	N	N	P	P	N	N
01DVA49	*K. pneumoniae*	FOX-like/SHV ESBL	P	P	N	N	P	P	N	N	P	P	N	N
01VUMM451	*K. pneumoniae*	DHA-like/SHV ESBL	P	P	N	N	P	P	P	P	P	P	P	P
01CSHS37	*K. pneumoniae*	FOX-like/SHV ESBL	P	P	N	N	P	P	N	N	P	P	N	P
01VCH72	*K. oxytoca*	DHA-like/SHV ESBL	N	N	P	N	N	N	P	P	N	N	P	P
01LSAI127	*K. oxytoca*	FOX-like	P	P	P	P	P	P	P	P	P	P	P	P
01LSAI141	*K. oxytoca*	FOX-like	P	P	P	N	P	P	P	P	P	P	P	P
01LSAI154	*K. oxytoca*	FOX-like	P	P	P	P	P	P	P	P	P	P	P	P
01CPMC7	*K. oxytoca*	FOX-like	N	N	N	N	P	N	P	N	P	P	P	P

[a] CL1, cefotetan plus LN-2-128, day 1; CL2, cefotetan plus LN-2-128, day 2; FL1, cefoxitin plus LN-2-128, day 1; FL2, cefoxitin plus LN-2-128, day 2; CR1, cefotetan plus 48-1220, day 1; CR2, cefotetan plus 48-1220, day 2; FR1, cefoxitin plus 48-1220, day 1; FR2, cefoxitin plus 48-1220, day 2; CS1, cefotetan plus Syn 2190, day 1; CS2, cefotetan plus Syn 2190, day 2; FS1, cefoxitin plus Syn 2190, day 1; FS2, cefoxitin plus Syn 2190, day 2; P, positive; N, negative.

[b] Resistance mechanism of interest; other β-lactamases may also be present in the isolate.

class A β-lactamases in addition to AmpC β-lactamases (4, 22). A novel AmpC β-lactamase inhibitor, Syn 2190 (Naeja Pharmaceutical Inc., Edmonton, Alberta, Canada), does not inhibit class A β-lactamases (18). LN-2-1220 is a C-3-substituted cephalosporin-derived inhibitor with a broad spectrum of inhibition and inhibits both class A (e.g., TEM and SHV) and class C (e.g., AmpC) β-lactamases, 48-1220 is a 2β alkenyl penicillanic acid sulfone inhibitor also with a broad spectrum of inhibition, and Syn 2190 is a monobactam derivative containing 1,5-dihydroxy-4-pyridone as the C-3 side chain and is a potent inhibitor of class C β-lactamases. A follow-up study was conducted to further evaluate the utility of cefotetan and cefoxitin alone and in combination with the AmpC β-lactamase inhibitors LN-2-128 and 48-1220, in addition to the novel AmpC β-lactamase inhibitor Syn 2190, to detect plasmid-mediated AmpC β-lactamases in clinical strains of *Klebsiella* spp.

In the present study, 51 recent clinical strains of *Klebsiella* spp. collected from various U.S. hospitals since 2001 were investigated using the previously reported inhibitor method for detection of plasmid-mediated AmpC β-lactamases (2) and also using the inhibitor Syn 2190. The aims were to extend the evaluation reported in our previous study by testing an expanded collection of strains and also to assess the diagnostic utility of Syn 2190 for AmpC detection. The strains included *K. pneumoniae* and *Klebsiella oxytoca* strains all having nonsusceptible cefoxitin MICs of ≥16 μg/ml according to CLSI criteria (16). The strains had all been previously characterized by an isoelectric focusing overlay technique that provided information about the number of β-lactamases produced and their isoelectric points and qualitative substrate and inhibitor profile information and/or multiplex PCR designed to detect different types of plasmid-mediated AmpC β-lactamases. Thirty-three isolates were found to produce the following plasmid-mediated AmpC β-lactamases: ACT-like ($n = 1$), FOX-like ($n = 30$), and DHA-like ($n = 2$) (J. A. Black, E. S. Moland, A. Hossain, T. J. Lockhart, L. Olson, K. S. Thomson, and N. Hanson, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-2034, 2003). In addition, 18 were found to produce the following class A β-lactamases: SHV-derived ESBLs ($n = 14$), KPC-2 ($n = 1$), and K1 ($n = 3$) (E. S. Moland, J. A. Black, N. Hanson, A. Hossain, B. Abdalhamid, W. Song, T. Lockhart, L. Olson, and K. Thomson, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-46, 2003) (Tables 1 and 2). Two well-characterized clinical strains of *K. pneumoniae* from South Africa also having nonsusceptible cefoxitin MICs of ≥16 μg/ml due to reduced outer membrane permeability

TABLE 2. Results of tests with strains not producing plasmid-mediated AmpC β-lactamases

Strain	Organism	Resistance mechanism[a]	Result[b] for:											
			CL1	CL2	FL1	FL2	CR1	CR2	FR1	FR2	CS1	CS2	FS1	FS2
01BH79	*K. pneumoniae*	KPC-2/SHV ESBL	P	P	N	N	N	N	N	N	N	N	N	N
01FH55	*K. pneumoniae*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01SUN70	*K. pneumoniae*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01UNMC52	*K. pneumoniae*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01JMH41	*K. pneumoniae*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01CMH44	*K. pneumoniae*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01VUMM72	*K. pneumoniae*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01MGH225	*K. pneumoniae*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01UW53	*K. pneumoniae*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01TU33	*K. pneumoniae*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01IFH60	*K. pneumoniae*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
Kleb189	*K. pneumoniae*	OMP^{P}/SHV-2 ESBL	N	N	N	N	N	N	N	N	N	N	N	N
Kleb192	*K. pneumoniae*	OMP	N	N	N	N	N	N	N	N	N	N	N	N
01ACH101	*K. oxytoca*	High K1	N	N	N	N	N	N	N	N	N	N	N	N
01AMLC70	*K. oxytoca*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01CCF87	*K. oxytoca*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01RH7	*K. oxytoca*	High K1	N	N	N	N	N	N	N	N	N	N	N	N
01TUHC90	*K. oxytoca*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N
01EUH162	*K. oxytoca*	High K1	N	N	N	N	N	N	N	N	N	N	N	N
01TU34	*K. oxytoca*	SHV ESBL	N	N	N	N	N	N	N	N	N	N	N	N

[a] Resistance mechanism of interest; other β-lactamases may also be present in the isolate.
[b] CL1, CL2, FL1, FL2, CR1, CR2, FR1, FR2, CS1, CS2, FS1, FS2, P, and N are as defined for Table 1.

were also included in the study (J. D. D. Pitout, E. S. Moland, and C. C. Sanders, Abstr. 36th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C39, 1996). Some plasmid-mediated AmpC β-lactamase-producing strains also produced SHV- and/or TEM-derived ESBLs (Tables 1 and 2). Strains Misc 304 and UMJMH14, producing the plasmid-mediated AmpC β-lactamases MIR-1 and DHA-1, respectively, were the positive controls, while Kleb 196, a porin mutant, and Kleb 116, producing the class A β-lactamase SHV-5, were the negative controls for this study. These were all selected from the previous inhibitor study (2).

Cefoxitin MICs were determined by CLSI microdilution methodology using a TREK frozen panel (17). Inhibition zones were determined by CLSI disk diffusion methodology on Mueller-Hinton II agar (Becton Dickinson, Sparks, MD) (15). Antibiotic disks tested were 30 μg cefotetan and 30 μg cefoxitin (Becton Dickinson, Sparks, MD) alone and in combination with either 20 μg LN-2-128, 20 μg 48-1220, or 150 μg Syn 2190. The quantities of the different inhibitors considered to be sufficient to inhibit AmpC β-lactamases were determined using positive and negative control strains described in a previous study (2). A positive test for a plasmid-mediated AmpC β-lactamase was an increase of ≥4 mm in zone diameter in the presence of an inhibitor compared to testing the antibiotic alone. The clinical strains were given numbers so the reader of the inhibition zones was blind as to which strains produced plasmid-mediated AmpC β-lactamases and which strains produced the class A β-lactamases. All strains were tested twice, on separate days (day 1 and day 2), to evaluate the reproducibility of the test results.

Results of inhibition tests for days 1 and 2 with cefoxitin-nonsusceptible *Klebsiella* spp. are shown in Tables 1 and 2. On day 1 in tests with cefotetan, LN-2-128 yielded positive tests with 25 of 33 of the known plasmid-mediated AmpC β-lactamase producers, 48-1220 yielded positive tests with 24 of 33, and Syn 2190 yielded positive tests with 30 of 33 (Table 1). On day 1 in tests with cefoxitin, LN-2-128 yielded positive tests with 8 of 33 of the known plasmid-mediated AmpC β-lactamase producers, 48-1220 yielded positive tests with 10 of 33, and Syn 2190 yielded positive tests with 17 of 33. With the strains not producing AmpC β-lactamases, all combinations of inhibitors with cefotetan and cefoxitin were negative except for cefotetan in combination with LN-2-128, which gave a positive result with 01BH79 (Table 2).

On day 2 in tests with cefotetan, LN-2-128 yielded positive tests with 18 of 33 of the known plasmid-mediated AmpC β-lactamase producers, 48-1220 yielded positive tests with 14 of 33, and Syn 2190 yielded positive tests with 30 of 33 (Table 1). On day 2 in tests with cefoxitin, LN-2-128 yielded positive tests with 6 of 33 of the known plasmid-mediated AmpC β-lactamase producers, 48-1220 yielded positive tests with 9 of 33, and Syn 2190 yielded positive tests with 18 of 33 (Table 1). Again, with the strains not producing plasmid-mediated AmpC β-lactamases, all combinations of inhibitors with cefotetan and cefoxitin were negative except for cefotetan in combination with LN-2-128, which gave a positive result with 01BH79 (Table 2).

Reproducibility was determined by comparing the results obtained on day 1 with results from day 2. In tests using LN-2-128, nine tests with cefotetan and four tests with cefoxitin were not reproducible. In tests using 48-1220, 12 tests with cefotetan and 3 tests with cefoxitin were not reproducible. In tests using Syn 2190, all tests with cefotetan were reproducible while five tests with cefoxitin were not reproducible.

Pai et al. compared the clinical features of patients infected by *K. pneumoniae* producing plasmid-mediated AmpC β-lactamases with isolates producing TEM- or SHV-related ESBLs and reported that those infected with plasmid-mediated AmpC-producing strains had similar clinical features and outcomes to those patients infected with ESBL producers (19).

Therefore, in vitro susceptibility testing of the expanded-spectrum cephalosporins may be unreliable for *Klebsiella* spp. producing plasmid-mediated AmpC β-lactamases. There is a need for a clinical microbiology laboratory to distinguish *Klebsiella* spp. producing plasmid-mediated AmpC β-lactamases from strains with other mechanisms responsible for nonsusceptibility to the expanded-spectrum cephalosporins and cephamycins.

Using the combination of Syn 2190 with cefotetan, the inhibitor-based test method had 91% sensitivity, 100% specificity, and 100% reproducibility. Syn 2190 performed very well with the clinical strains and showed potential of using an AmpC β-lactamase inhibitor for detection of *Klebsiella* spp. producing plasmid-mediated AmpC β-lactamases. The isolates of *Klebsiella* spp. used in our study produced FOX, ACT, and DHA types of AmpC β-lactamases. In additional tests, the combination of Syn 2190 and cefotetan also yielded positive results with four strains of *Proteus mirabilis* and six strains of *Salmonella* spp. producing CMY types of AmpC β-lactamases (data not shown). This suggests that Syn 2190-based tests have the potential to detect a wide range of AmpC β-lactamases. We recommend that cefotetan in combination with Syn 2190 be used to detect *K. pneumoniae* producing plasmid-mediated AmpC β-lactamases.

We report a study that evaluated the utility of cefotetan and cefoxitin alone and in combination with the AmpC β-lactamase inhibitors LN-2-128, 48-1220, and Syn 2190, to detect cefoxitin nonsusceptible clinical strains of *Klebsiella* spp. producing well-characterized plasmid-mediated AmpC β-lactamases. Our results showed that inhibitor based disk tests using LN-2-128 and 48-1220 were not reproducible and detected fewer of these strains than Syn 2190 (Table 1).

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