Performance of the Phoenix bacterial identification system compared with disc diffusion methods for identifying extended-spectrum *β*-lactamase, AmpC and KPC producers

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Phenotypic identification of AmpC, KPC and extended-spectrum *β*-lactamases (ESBLs) among members of the *Enterobacteriaceae* remains challenging. This study compared the Phoenix Automated Microbiology System (BD Diagnostics) with the Clinical and Laboratory Standards Institute confirmatory method to identify ESBL production among 200 *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates. The Phoenix system misclassified nearly half of the isolates as ESBL-positive, requiring manual testing for confirmation. Inclusion of aztreonam ± clavulanic acid (CA) and cefpodoxime ± CA in the testing algorithm increased the ESBL detection rate by 6 %. Boronic acid-based screening identified 24 isolates as AmpC[+], but in a subset of genotypically characterized isolates, appeared to have a high false-positivity rate. PCR screening revealed eight KPC[+] isolates, all of which tested as ESBL[+] or ESBL[+] AmpC[+] by phenotypic methods, but half were reported as carbapenem-susceptible by the Phoenix system. Overall, these results indicate that laboratories should use the Phoenix ESBL results only as an initial screen followed by confirmation with an alternative method.

INTRODUCTION

Extended-spectrum *β*-lactamases (ESBLs), AmpC and KPC *β*-lactamases are important mechanisms of *β*-lactam resistance among members of the *Enterobacteriaceae*. Accurate detection of *β*-lactamase producers is critical for therapeutic and infection control decision-making. The Clinical and Laboratory Standards Institute (CLSI, 2008) has standardized methods for ESBL detection, but many laboratories rely on automated systems for antimicrobial susceptibility testing. Unfortunately, some automated systems have difficulty detecting ESBLs, AmpCs and KPCs (Tenover *et al.*, 2006; Wiegand *et al.*, 2007). With the recent implementation of the Phoenix Automated Microbiology System (BD Diagnostics), we observed a higher frequency of ESBL[+] calls among *Escherichia coli* and *Klebsiella pneumoniae* isolates compared with the CLSI disc diffusion (DD) method.

Incorporation of manual methods in an ESBL-testing algorithm for laboratories using automated instruments has not been well delineated. To determine the optimal algorithm, we (i) compared the CLSI and Phoenix methods for identifying ESBL[+] isolates of *E. coli* and *K. pneumoniae*, (ii) assessed the value of an extended phenotypic method [cefpodoxime and aztreonam ± clavulanic acid (CA)] for enhancing ESBL detection, (iii) evaluated boronic acid (BA) with cefotetan or ceftazidime ± CA to identify AmpC producers that could mimic or mask ESBL production, and (iv) assessed these phenotypic tests with KPC-positive clinical isolates.

METHODS

Bacterial isolates. Two hundred isolates of *E. coli* (*n*=119) and *K. pneumoniae* (*n*=81) were collected at two sites representing urban (The Johns Hopkins Hospital, Baltimore, MD, USA) and geographically diverse (ARUP Laboratories, Salt Lake City, UT, USA) populations. Identification and susceptibility testing were performed

Abbreviations: BA, boronic acid; CA, clavulanic acid; DD, disc diffusion; ESBL, extended-spectrum *β*-lactamase.

on a Phoenix system (instrument version 5.15A, software version 5.10A/V4.31A), using a standard protocol, at both sites. One hundred consecutive isolates flagged as ESBL$^+$ by the Phoenix system and 93 randomly selected ESBL$^-$ isolates were collected. Seven isolates resistant to a third-generation cephalosporin or aztreonam but called ESBL$^-$ by the Phoenix system were selected for inclusion.

Phenotypic identification of ESBL and AmpC producers. All Phoenix ESBL$^+$ and ESBL$^-$ isolates were tested by DD for ESBL (CLSI, 2008) and AmpC production as described previously (Coudron, 2005). Discs containing antibiotics [30 µg ceftazidime, 30 µg cefotaxime, 10 µg cefpodoxime, 30 µg aztreonam (BD Diagnostics) or 30 µg cefotetan (Remel)] with or without β-lactamase inhibitors [10 µg CA (GlaxoSmithKline) or 400 µg BA (Sigma)] were placed on Mueller–Hinton agar streaked with the test organism and incubated at 35 °C for 16–18 h in ambient air. In total, 12 drug/inhibitor combinations (ceftazidime $\pm$ CA and cefotaxime $\pm$ CA for the CLSI ESBL test, cefpodoxime $\pm$ CA and aztreonam $\pm$ CA for the ESBL test, cefotetan $\pm$ BA for AmpC detection, and ceftazidime + BA $\pm$ CA for AmpC-masked ESBL detection) were tested for each isolate. Results were considered positive (indicative of β-lactamase) for any drug showing a $\geqslant 5$ mm increase in zone of inhibition with an inhibitor.

Molecular characterization of resistance mechanisms. A random subset of 24 isolates determined as ESBL$^+$ by the Phoenix system were examined for ESBL, AmpC and KPC β-lactamases by IEF, PCR and sequencing to confirm the phenotypic findings. Seven isolates determined as ESBL$^-$ by the Phoenix assay but ESBL$^+$ and/or AmpC$^+$ by DD were also characterized by molecular methods.

IEF was performed by loading 10 µl of a crude enzyme extract onto pre-cast gels (Ampholine PAGplate, pH 3.5–9.5; GE Healthcare). Purified TEM and SHV enzymes and isolates with previously characterized β-lactamases were used as controls (Paterson *et al.*, 2001, 2003). PCR amplification was performed on 10 µl aliquots of diluted lysates from overnight cultures using primers specific for bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{PER}, bla_{ACT}, bla_{MIR}, bla_{CMY}, bla_{BIL}, bla_{LAT} and bla_{DHA} genes as described previously (Paterson *et al.*, 2003). DNA sequencing was performed (ALFexpress, Thermo Sequenase; GE Healthcare) to identify TEM and SHV ESBLs by comparison with previously characterized enzymes (http://www.lahey.org/Studies) and to subtype KPC$^+$ isolates. This group of 31 well-characterized isolates served as a reference set by which to compare phenotypic methods. In addition, all 200 isolates were screened by PCR for the bla_{KPC} gene as described previously (Smith Moland *et al.*, 2003).

RESULTS AND DISCUSSION

ESBL detection

Of 100 isolates determined as ESBL$^+$ by the Phoenix system, only 51 were verified by the CLSI confirmatory method (Table 1). Even the extended testing algorithm, including cefpodoxime $\pm$ CA and aztreonam $\pm$ CA, confirmed only 54 % of the isolates determined as ESBL$^+$ by the Phoenix system. Of 100 isolates determined as ESBL$^-$ by the Phoenix test (60 *E. coli* and 40 *K. pneumoniae*), 96 % were confirmed by the DD methods (Table 1).

Molecular analyses on a subset of isolates confirmed the DD results for identification of ESBLs. Twenty-seven of 31 isolates were concordant between DD and molecular testing, whereas only nine (29 %) were concordant between the molecular and Phoenix ESBL results (Table 2). Two

Table 1. Summary of phenotypic ESBL and AmpC classification of 200 clinical isolates categorized as ESBL$^+$ ($n=100$) or ESBL$^-$ ($n=100$) by the Phoenix system

Results in parentheses show the number of *E. coli* and *K. pneumoniae*, respectively.

Phenotype*	ESBL$^+$ by Phoenix	ESBL$^-$ by Phoenix
ESBL$^+$	46 (19, 27)†	2 (1, 1)
ESBL$^+$ AmpC$^+$	8 (1, 7)‡	2 (0, 2)§
AmpC$^+$	11 (11, 0)	3 (2, 1)
ESBL$^-$ AmpC$^-$	35 (28, 7)	93 (57, 36)
Total	100 (59, 41)	100 (60, 40)

*Determined by DD testing as described in the text.
†One *E. coli* and one *K. pneumoniae* were determined as ESBL$^+$ only by the extended method.
‡One *K. pneumoniae* was determined as ESBL$^+$ only by the extended method.
§Both isolates were determined as ESBL$^+$ only by the extended method.

isolates (101 and 118) were determined as ESBL$^+$ by DD and Phoenix testing but were ESBL$^-$ KPC$^+$ by PCR. This was not unexpected as KPC enzymes are CA-sensitive and can behave phenotypically like ESBLs (Jacoby & Munoz-Price, 2005). Of seven isolates determined as ESBL$^-$ by the Phoenix system but ESBL$^+$ and/or AmpC$^+$ by DD testing, the molecular results agreed with the DD results in every case. Overall, the molecular data supported the DD results, indicating that the Phoenix system was overcalling ESBLs.

Our data confirm the high sensitivity (93 %) but questionable specificity (68 %) reported for Phoenix ESBL detection (Thomson *et al.*, 2007; Wiegand *et al.*, 2007). Because isolates producing false-positive and false-negative results in the CLSI test have been described (Thomson *et al.*, 2007), it is not considered the gold standard for ESBL detection. However, it is a robust assay that is the mainstay of ESBL testing in many laboratories (Steward *et al.*, 2001; Tenover *et al.*, 2003). Our molecular data support the CLSI results and suggest that it is unlikely that the CLSI-confounding isolates were present in sufficient numbers to explain the 49 discrepant ESBL calls. The extended DD method increased the detection of ESBLs by 6 % compared with the traditional CLSI method, which may warrant adoption in areas with high ESBL prevalence. Based on these results, we recommend the Phoenix ESBL test be used only as an initial screen for ESBLs, followed by confirmation with an alternative test such as the CLSI method or a molecular assay.

AmpC detection

DD testing with BA as an inhibitor identified 24 putative AmpC$^+$ isolates, including 10 AmpC$^+$ ESBL$^+$ isolates (Table 1). Only one isolate, 101, showed an AmpC-masked

Table 2. Genotypic and phenotypic β-lactamase results from 31 selected isolates

Isolate no.	Species	Genotype*			Phoenix ESBL†	DD‡	
		ESBL	AmpC	KPC		ESBL	AmpC
A10	E. coli	−	−	−	+	−	+
A13	E. coli	−	−	−	+	−	−
A15	E. coli	−	+	−	+	−	+
D2	K. pneumoniae	−	−	−	+	+	+
E6	E. coli	CTX-M	−	−	+	+	−
F1	K. pneumoniae	−	+	−	−	−	+
F4	E. coli	−	+	−	−	−	+
F6	E. coli	CTX-M	−	−	−	+	−
F9	K. pneumoniae	SHV-28, CTX-M	−	−	+	+	−
G7	K. pneumoniae	−	−	−	+	−	−
H2	K. pneumoniae	SHV-12	+	−	−	+	+
J9	K. pneumoniae	SHV-12	+	−	−	+	+
J10	K. pneumoniae	SHV-12	−	−	−	+	−
101	K. pneumoniae	−	−	+	+	+§	+
104	E. coli	−	−	−	+	−	−
109	E. coli	−	−	−	+	−	−
113	E. coli	−	−	−	+	−	+
116	E. coli	−	−	−	+	−	−
118	K. pneumoniae	−	−	+	+	+	+
119	K. pneumoniae	SHV-12	−	−	+	+	−
124	K. pneumoniae	−	−	−	+	−	−
126	E. coli	CTX-M	−	−	+	+	+
140	E. coli	CTX-M	−	−	+	+	−
141	E. coli	−	−	−	+	−	−
144	E. coli	−	−	−	+	−	+
146	E. coli	−	−	−	+	−	−
147	K. pneumoniae	CTX-M	−	−	+	−	−
154	K. pneumoniae	−	−	−	+	−	−
166	K. pneumoniae	−	−	−	+	−	−
169	K. pneumoniae	−	−	−	+	−	−
2-9	E. coli	−	+	−	−	−	+

*TEM, SHV and KPC PCR products were sequenced to identify *bla* subtypes. Both KPC$^+$ isolates were type 3 based on sequence analysis.
†ESBL results determined using the Phoenix system.
‡Phenotypes were determined by DD testing as described in the text.
§This isolate was determined as ESBL only using ceftazidime$+$BA$\pm$CA.

ESBL phenotype (increased susceptibility to ceftazidime only with both BA and CA). Molecular analyses revealed that Phoenix and DD testing misidentified this KPC-3 producer as ESBL$^+$ and ESBL$^+$ AmpC$^+$, respectively (Table 2). This is not surprising as KPCs can be sensitive to both CA and BA inhibition (Jacoby & Munoz-Price, 2005; Livermore *et al.*, 2007; Pasteran *et al.*, 2008). Of the 10 isolates determined as AmpC$^+$ ESBL$^+$ by BA and CA inhibition, the CLSI and Phoenix methods classified 70 and 80 % as ESBL$^+$, respectively. Of the 14 AmpC$^+$ ESBL$^-$ isolates, only 3 (21 %) were correctly determined as ESBL$^-$ by the Phoenix system (Table 1).

Molecular testing did not correlate well with DD results for AmpC detection. Only six of the 13 phenotypically AmpC$^+$ isolates were found to be AmpC$^+$ by molecular testing (Table 2), suggesting that the BA method overcalled

AmpC producers among these isolates. A limitation of using BA to identify AmpC producers is that it does not inhibit AmpC hydrolysis of all cephalosporins (Jacoby *et al.*, 2006; Yagi *et al.*, 2005). However, cefotetan$\pm$BA, as used here, previously detected nearly 90 % of AmpC enzymes in control strains (Coudron, 2005). The AmpC primers we used were specific for only a subset of AmpC genes (Paterson *et al.*, 2003), which may explain discrepancies between phenotypic and molecular data for our 31 reference isolates. A broader set of primers, such as those used by Pérez-Pérez & Hanson (2002), may confirm more AmpC$^+$ isolates. Given the current data, including confirmation that KPC producers can be sensitive to BA, like AmpCs (isolate 101, Table 2), we cannot recommend cefotetan$\pm$BA as a stand-alone test for AmpC detection.

Table 3. Cephalosporin and carbapenem susceptibility and MIC data from KPC$^+$ isolates

All isolates were *K. pneumoniae* and categorized as ESBL$^+$ using the Phoenix system.

Isolate no.	DD interpretation*					MIC (µg ml^{-1})†				Phenotype‡
	Atm	Ctt	Cpd	Ctx	Caz	Cro	Fep	Ipm	Mem	
101	R	R	R	R	R	>32	>16	>8	>8	ESBL + AmpC
107	R	S	R	S	I	32	2	<=1	<=1	ESBL
117	I	S	R	S	I	32	2	8	<=1	ESBL
118	R	R	R	R	R	>32	4	<=1	<=1	ESBL + AmpC
123	R	S	R	R	R	>32	>16	<=1	4	ESBL + AmpC
155	R	S	R	R	R	>32	>16	4	>8	ESBL + AmpC
167	R	R	R	R	R	>32	>16	>8	>8	ESBL + AmpC
171	R	S	R	I	R	>32	8	<=1	<=1	ESBL

*Interpretations based on zone size using CLSI criteria. S, Susceptible; I, intermediate susceptibility; R, resistant. Atm, aztreonam; Ctt, cefotetan; Cpd, cefpodoxime; Ctx, cefotaxime; Caz, ceftazidime.
†MICs were determined by the Phoenix system. Bold indicates resistance and underlining indicates intermediate susceptibility according to CLSI criteria. Cro, Ceftriaxone; Fep, cefepime; Ipm, imipenem; Mem, meropenem.
‡Phenotypes were determined by DD testing as described in the text.

KPC detection

A PCR screen of our 200 clinical isolates revealed eight carrying the bla_{KPC} gene. Although both phenotypic methods indicated β-lactamase production (ESBL and/or AmpC) in these isolates, the Phoenix system unfortunately reported half as meropenem- and imipenem-susceptible (Table 3). Many automated instruments fail to detect carbapenemases, which is a serious concern in the laboratory (Tenover *et al.*, 2006). Ertapenem may be a more sensitive indicator of KPC production, but was not included on the Phoenix panels used in this or a previous study (Anderson *et al.*, 2007). Overall, these observations highlight the significant presence of KPC$^+$ isolates in the USA and the need for more reliable detection methods.

Conclusions

From 100 isolates, the Phoenix system made four false-negative and 46 false-positive ESBL calls relative to DD testing. Of the 20 discrepants tested by molecular methods, DD results were confirmed for all four false-negatives and 94 % of false-positives. In addition, half of the KPC$^+$ isolates were reported as carbapenem-susceptible by the Phoenix system. These errors can adversely impact on the healthcare system in a number of ways, including inappropriate therapy and inaccurate antimicrobial surveillance data.

These results show that the Phoenix system overcalled ESBLs relative to the CLSI confirmatory and molecular testing. Neither the Phoenix system nor DD testing appear to be robust enough for routine detection of AmpC$^+$ or KPC$^+$ isolates, stressing the need for reliable methods to detect these important resistance mechanisms in the clinical laboratory.

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