Detection of ESBL and/or plasmid-mediated AmpC β-Lactamase enzymes in *Enterobacteriaceae* using MASTDISCS™ ID AmpC and ESBL Detection Discs and to compare this with current laboratory detection methods.

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INTRODUCTION

The β-lactamases are the most significant group of enzymes involved in conferring resistance to β-lactam antibiotics in gram-negative bacteria. They work by hydrolysing the β-lactam bond of any number of substrates thus rendering the antibiotic ineffective. Within months of the broader-spectrum β-lactam ampicillin being released in Europe in 1964, the first incidence of resistance to ampicillin in *Escherichia* coli was described. Today there are many β-lactam antibiotics and many more β-lactamase enzymes, including Extended Spectrum β-Lactamases (ESBL's) and plasmid-mediated AmpC β-Lactamases.

Extended Spectrum β-Lactamase (ESBL) enzymes were discovered in the early 1980's and the term coined in 1988 to describe the new resistance patterns that extended beyond the broad-spectrum β-lactamases. Traditionally ESBL's have been defined as enzymes that are inhibited by clavulanic acid and that have activity against extended spectrum cephalosporins. They are classed by the Ambler structural classification as molecular class A and by the Bush-Jacoby-Medeiros functional classification as functional class 2be. ESBL's generally confer resistance to penicillins, cephalosporins and monobactams but don't hydrolyse the cephamycins (cefoxitin and cefotetan) and are inhibited by β-Lactamase inhibitor combinations such as clavulanic acid, sulbactam and tazobactam.

AmpC β-Lactamases first appeared in the late 1970's as an inducible resistance in organisms that would overproduce their chromosomal *ampC* gene, probably due to the use of cephamycins and the introduction of β-lactamase inhibitor combinations. These are known as derepressed mutants and mainly form the group often referred to as ESCHAPPM's. *Escherichia coli* and *Shigella sonnei* contain a chromosomal *ampC* gene but due to a lack of the regulatory gene *ampR*, the *ampC* gene is not expressed in amounts large enough to confer resistance. AmpC β-lactamases are usually resistant to penicillins, cephalosporins including the cephamycins and monobactams, are resistant to β-lactamase inhibitor combinations but are usually sensitive to the carbapenems. AmpC β-lactamases are Ambler molecular class C and Bush-Jacoby-Medeiros functional class 1.

The first plasmid-mediated AmpC β-lactamase was isolated in 1988 from a *Klebsiella pneumoniae*. *Klebsiella* spp. along with *Salmonella* spp. and *Proteus mirabilis* are likely sources of plasmid-mediated AmpC β-lactamases and are good for identification as they lack a chromosomal *ampC* gene. *Escherichia coli*, although a likely source also, is problematic as its low-level *ampC* expression can become hyper produced. Currently there are over 40 known plasmid-mediated AmpC β-lactamases derived from *Enterobacter cloacae*, *Morganella morganii*, *Hafnia alvei*, *Citrobacter freundii* and other unknown sources. The chromosomal *ampC* gene of *E.coli* has not been found on a plasmid or other transferable element and thus allows hyper production of the *E.coli ampC* gene be distinguished from an *E.coli* with a plasmid-mediated AmpC β-lactamase. The plasmid-mediated AmpC β-lactamases confer resistance similar to their chromosomal counterparts.

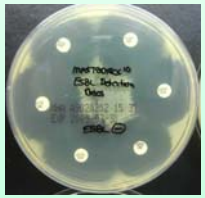
Current detection methods for ESBL's include phenotypic disc detection i.e. double-disc synergy test (DDST – keyhole phenomenon) and molecular based assays. Molecular detection, while the gold standard, may not be practical for many laboratories due to many different genes involved of which all could not possibly be included in one single test. Phenotypic disc diffusion methods are the preferred method for detection of ESBL's in most laboratories due to their relative ease and sensitivity and their low cost.

Current plasmid-mediated AmpC β-lactamase detection methods include an insusceptibility screen using a cephamycin (usually cefoxitin) disc. This is not 100% accurate as reduced outer membrane permeability can also cause insusceptibility. Also the Amp-C Class (ACC)-like plasmid-mediated AmpC β-lactamases, are susceptible or weakly insusceptible to cefoxitin and will not be detected by the screen. Cloxacillin is an effective inhibitor of AmpC β-lactamases and thus can be used as a substitute for clavulanate in the DDST. The problem arises, as with the standard test, that ineffective spacing of discs can lead to incorrect results. A plasmid-mediated AmpC β-lactamase Multiplex PCR has been described but is limited to current known transferable *ampC* genes.

The aim of this study was to evaluate MASTDISCS™ ID AmpC and ESBL detection discs with current laboratory detection methods. The implications of incorrect diagnosis of either ESBL's or plasmid-mediated AmpC β-lactamases are far-reaching. Incorrect treatment and treatment failure can occur as well as a failure to isolate a potential infection control risk for a health care setting.

METHODS

- All *Enterobacteriaceae* isolated in the laboratory undergo screening with a Ceftazidime Screen Test (CST) (containing 0.5µg/mL ceftazidime) and a DDST, incorporated into routine susceptibility testing, with amoxicillin/clavulanate adjacent to cefotaxime.
- Insusceptibility to cefpodoxime and cefoxitin and a further DDST with cefpodoxime adjacent to amoxicillin/clavulanate is also included for Urinary isolates.
- If any of these are positive a further two DDST's are set up with cefipime adjacent to ticarcillin/clavulanate adjacent to aztreonam and insusceptibility to cefoxitin is tested for non-urinary isolates.
- If keyholes were apparent on any of the DDST's, MASTDISCS™ ID ESBL and a MASTDISCS™ ID AmpC and ESBL detection discs were set up for analysis.
- If organisms presented with no apparent or doubtful keyholes but cefpodoxime was insusceptible, cefotaxime and aztreonam had reduced zone diameters (≤27mm), there was growth on CST and/or cefoxitin was insusceptible then MASTDISCS™ ID ESBL and MASTDISCS™ ID AmpC and ESBL detection discs were set up for analysis.
- All disc diffusion testing performed according to CLSI procedure, equal in turbidity to a 0.5 McFarland standard and set up on Biomerieux Australia Mueller Hinton Agar (CLSI Formulation).



If MASTDISCS™ ID ESBL Detection Discs are placed with the cephalosporin next to cephalosporin/clavulanate, and appropriately spaced, this test can be used as both the combined disc test and the double-disc synergy test.

RESULTS

Both MASTDISCS™ ID ESBL Detection Discs and MASTDISCS™ ID AmpC and ESBL Detection Discs use a 5 mm zone increase of a combination disc plus inhibitor compared to a cephalosporin only disc as an indicator of an ESBL and/or AmpC.

Sensitive | ESBL positive | AmpC positive | ESBL & AmpC positive

MASTDISCS™ ID ESBL and AmpC Detection Discs

A total of 2472 *Enterobacteriaceae* samples isolated from the SVH Microbiology Department were tested. Of all detection methods available, including screen tests, DDST's and MASTDISCS™ ID Detection discs a total of 64 new ESBL's were isolated.

Total ESBL's (inclusive of all methods) = 64

Detection Discs		DDST – Keyhole Phenomenon			CST Plate Growth		
ESBL	AmpC and ESBL	1st Line	2nd Line	1st & 2nd combined	Negative	Positive	Negative
60	**63**	**42**	**58**	**58**	**6**	**46**	**17**

Total Plasmid-Mediated AmpC's = 11

	E.coli	*K.oxytoca*	*K.pneumoniae*
FOX = R	**8**	**1**	**1**
FOX = S	**1**	**0**	**0**
Total	**9**	**1**	**1**
ESBL Positive	**None**	**None**	**1**

A total of 11 plasmid-mediated AmpC β-lactamases were detected using a Cefoxitin (FOX) insusceptibility screen and MASTDISCS™ ID ESBL and AmpC Detection Discs.

Total ESCHAPPM's* Tested (Inducible Chromosomal AmpC's) = 20

ESBL's	No Transferable Resistance	AmpC Negative+	Cefoxitin Resistant	Cefoxitin Sensitive
12	**8**	**7**	**19**	**1#**

* Tested using both sets of MASTDISCS™ ID Detection Discs.
\+ AmpC Negative by MASTDISCS™ ID ESBL and AmpC Detection Discs
\# Expected to be Cefoxitin sensitive, *Hafnia alvei*.

- 19 out of 39 non plasmid-mediated AmpC *E.coli*'s showed an increased zone size to disc 3 of AmpC Detection Discs ranging from 1mm to 8mm. This result is unexpected and not consistent with disc instructions.
- Of FOX resistant organisms, 13 were AmpC negative. 3 *E.coli*'s, 3 *Klebsiella* spp., 6 *Enterobacter* spp., 1 Serratia sp.
- Only 1 ESBL was detected by MASTDISCS™ ID ESBL Detection Discs and not MASTDISCS™ ID ESBL and AmpC Detection Discs.

DISCUSSION & CONCLUSION

- Of 64 ESBL's in total, the MASTDISC™ ID ESBL and AmpC Detection Discs (ESBL/AmpC discs) detected 63. The ESBL not detected by the ESBL/AmpC discs had an increase of 5mm to Ceftazidime 30µg (CAZ) in the MASTDISC™ ID ESBL Detection Discs (ESBL discs). The ESBL/AmpC discs do not contain CAZ, only Cefpodxime 10µg (CPD). This is most likely the reason it was missed and perhaps more cephalosporins could be developed for these discs.
- The ESBL/AmpC discs detected 98% of ESBL's and out-performed all other methods of detecting ESBL's in the laboratory by at least 4%.
- The ESBL discs detected 94% of ESBL's. The DDST's and the CST plates did not detect any ESBL's that the ESBL or ESBL/AmpC discs didn't detect. These screens are good indicators of ESBL's on primary testing.
- Of the 11 plasmid-mediated AmpC β-lactamases detected all but 1, an *E.coli*, were Cefoxitin resistant. This is perhaps an *ampc* hyper-producing *E.*coli. To properly identify transferable resistance opposed to hyper-production a multiplex PCR for plasmid-mediated AmpC genes is required.
- Low-level expression of *E.*coli's chromosomal *ampc* gene is the most probable reason for seeing increased zone sizes to disc 3 of the ESBL/Ampc discs. As long as users are aware of this and understand *E.coli*, it can be worked around.
- FOX resitant, AmpC negative *E.coli*'s and *Klebsiella* spp. confer this resistance by other mechanisms such as reduced outer-membrane permeability.
- When AmpC's were not detected in the ESCHAPPM's it is perhaps due to the *ampc* genes not being induced (expressed) and therefore the ESBL/AmpC discs don't detect them.
- Since AmpC β-lactamases are found chromosomally on many *Enterobacteriaceae* as well as on transferable elements it is very important to correctly identify the organism before calling an AmpC β-lactamase plasmid-mediated.

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