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Short Communication

Reliability of various antimicrobial susceptibility testing methods for piperacillin/tazobactam in challenging *Escherichia coli* isolates

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ABSTRACT

Objective: Piperacillin/tazobactam antimicrobial susceptibility testing (AST) against Enterobacterales can be challenging. The aim of this study was to assess the reproducibility of various automated (VITEK 2) and nonautomated AST methods (broth microdilution (BMD), minimum inhibitory concentration (MIC) test strip, and disk diffusion) for piperacillin/tazobactam in 'challenging' *E. coli* isolates.

Methods: We performed 20 repeated ASTs for seven clinical *E. coli* isolates: Two resistant to piperacillin/tazobactam but susceptible to amoxicillin/clavulanic acid, four isolates with various β-lactamase coding genes (two $bla_{\text{TEM-1}}$, one $bla_{\text{OXA-1}}$, and one with plasmidal bla_{ampC}), and one isolate where VITEK 2 initially could not produce MIC measurements for piperacillin/tazobactam (i.e. no results generated).

Results: Upon repetition, the same MIC as the mode value (i.e. the most frequent MIC value of each AST method) was found between 21% and 87% (BMD), 46% and 100% (VITEK 2), and 48% and 100% (gradient test) of the repetitions. The range of essential agreement percentage (i.e. $\pm$1 doubling dilution from this mode value) was 53–100% (BMD), 63–100% (VITEK 2), and 100% (gradient test). Percent categorical agreement (same susceptible of resistant category using EUCAST breakpoint v. 14.0) was 71–100% (BMD), 85–92% (VITEK 2), 76–100% (gradient test) and 100% (disk diffusion).

Conclusions: : In conclusion, this study provides insight on the reliability of AST results for piperacillin/tazobactam in challenging *E. coli* isolates. While the results indicate that most methods are generally reproducible, certain isolates may present inconsistent MIC results.

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1. Introduction

Reproducibility of antimicrobial susceptibility testing (AST) for piperacillin/tazobactam in Enterobacterales is challenging. Repeating AST on Enterobacterales isolates in the MERINO study at a centralised location using the broth microdilution (BMD) method revealed that 6% of previously susceptible isolates, as determined by various AST methods at participating laboratories, were resistant to piperacillin/tazobactam [1]. Recently, our laboratory reported a false-resistant result for piperacillin/tazobactam in *Escherichia coli* from an external quality control programme using an automatic AST system (VITEK 2, bioMérieux, Marcy l'Etoile, France). Upon repeating the AST, the result was susceptible. VITEK 2 also occasionally reported *E. coli* as resistant to piperacillin/tazobactam while susceptible to amoxicillin/clavulanic acid. This 'reversed' pattern may also indicate a false-resistant result for piperacillin/tazobactam, as the pattern of β-lactamase/β-lactamase inhibitor resistance in *E. coli* typically extends unidirectionally, from ampicillin/sulbactam to piperacillin/tazobactam, through amoxicillin/clavulanic acid [2]. Also in this case, repeating the AST often shows susceptibility to piperacillin/tazobactam. Repeating AST can thus reveal a susceptibility pattern that fits our expectation. However, without external quality control, prior knowledge on β-lactamase/β-lactamase inhibitor resistance patterns and molecular testing, such errors might go undiscovered. This raises the question of how often an AST method actually produces similar results when repeated almost without limit. Arguably, the problem is more enhanced in *E. coli* isolates with insufficient inhibition by β-lactamase inhibitors in the AST system, such as several TEM β-lactamases related to TEM-1 or TEM-2 that are associated with resistance to clavulanic acid (designated as inhibitor-resistant TEM) [3,4]. OXA-1 penicillinase [3,5] and plasmid AmpC

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β-lactamase [6,7] are other enzymes that exhibit weak affinity for β-lactamase inhibitors including tazobactam.

Understanding this consistency provides insight into the reliability of the results produced by various AST methods. Therefore, we investigated the reproducibility of various AST methods for piperacillin/tazobactam in *E. coli* by performing extensive repetition of AST in non-ESBL *E. coli* isolates in which we expected AST would be 'challenging'.

2. Materials and methods

2.1. Isolates

We selected seven *E. coli* isolates from routine practice in which AST may be challenging. They included two *E. coli* strains resistant to piperacillin/tazobactam but susceptible to amoxicillin/clavulanic acid (isolate nos. 26239 and 24535, both with the minimum inhibitory concentration (MIC) for amoxicillin/clavulanic acid of 4 mg/L). Polymerase chain reaction and sequence analysis did not reveal the β-lactamase gene in these strains. Over a 5-year period, such a pattern occurred in 30 out of 12 882 (0.23%) nonextended spectrum β-lactamases *E. coli* isolates isolated in our laboratory. Four *E. coli* isolates were selected for harbouring various β-lactamases-coding genes: two $bla_{\text{TEM-1}}$ (26664 and 26273), one $bla_{\text{OXA-1}}$ (00150), and one with plasmidal bla_{ampC} (21530). TEM β-lactamases were detected using real-time in-house polymerase chain reaction with primers and probes as described by Roschanski et al. [8] and further characterised using sequence analysis. OXA-1 was detected using sequence analysis. Additionally, one *E. coli* isolate (44782) was selected because the VITEK 2 piperacillin/tazobactam measurement initially failed. For each isolate, 20 repetitions were performed for each AST method (BMD, VITEK 2, gradient test, and disk diffusion methods), totalling 560 tests.

2.2. Microbiological procedures

After retrieval from freezer storage, the isolates were inoculated onto Columbia Agar with 5% sheep blood (BD, Heidelberg, Germany) and incubated overnight at 37 °C. To obtain fresh isolates for each AST repetition, this process was repeated daily as advised by EUCAST [9]. To perform ASTs, an inoculum of 0.5 McFarland turbidity standard was used. The same inoculum was employed for all AST methods in each repetition. EUCAST 15-15-15 rules were strictly followed. The same inoculum was used for all AST methods in each repetition.

All AST procedures were performed according to manufacturer instructions by one technologist. BMD was performed using custom-made plates produced according to ISO standard 20776-1, with piperacillin concentration ranges from 0.25 to 128 mg/L. A VITEK AST-N344 card was used for VITEK 2 with the piperacillin concentration range 2–48 mg/L. The range for the MIC test strip (Liofilchem, Roseto degli Abruzzi, Italy) for piperacillin concentration was between 0.006 and 256 mg/L. For BMD, VITEK 2, and the test strip, the tazobactam concentration was fixed at 4 mg/L. Additionally, disk diffusions using a piperacillin/tazobactam disk concentration of 30–6 µg was tested.

ASTs were read according to EUCAST BMD and the MIC test strip reading guide by the same technologist and another investigator based on photographs. The zone of inhibition was measured using a calliper from the back of the agar plate with reflected light according to EUCAST [9], and MIC results from VITEK 2 were noted. For all AST procedures, control strains according to EUCAST were used. All AST results, except for VITEK 2, were photographed and reassessed by a second reader.

2.3. Statistical analysis

Each isolate underwent 20 repetitions, with a maximum of three per day. The mode (i.e. most frequent value) of the MIC, determined by BMD, or VITEK 2 or MIC test strip, was used as the target value and categorised according to the EUCAST v. 14.0 breakpoint. We calculated the percent essential agreement (% EA) as ±1 MIC of the mode MIC value and percent categorical agreement (% CA) as the proportion of isolates in the same category (susceptible or resistant) as the mode value [10]. Very major error (VME) and major error (ME) were also determined. VME occurs when the repetitions show as susceptible while the mode value is reported as resistant, and ME occurs when the mode value is reported as susceptible while repetitions are reported as resistant [10].

We also explored the agreement between various AST methods for these isolates using the susceptible or resistant category of MIC according to BMD as the reference standard, following ISO 20776-2:2021 guidelines. For disk diffusion, we calculated the mean with standard deviation (SD), median with range, VME, and ME. Last, we calculated the % EA and % CA of various ASTs using the MIC mode value of BMD, interpreted by following EUCAST v. 14.0 breakpoint as the reference standard.

Inter-reader reproducibility of BMD and MIC test strip readings, expressed as a percentage of ±1 MIC values by two readers, was also computed.

3. Results

3.1. Reproducibility of results for piperacillin/tazobactam for each AST method

Upon repetition, we found the same MIC as the mode value (i.e. the target value) of the MIC between 21% and 87% (BMD), 46% and 100% (VITEK 2), and 48% and 100% (gradient test) (Table 1). The range of % EA, i.e. ±1 doubling dilution from this mode value, was between 53% and 100% (BMD), 63% and 100% (VITEK 2), and 100% (gradient test). % CA using EUCAST breakpoint v. 14.0 ranged from 71% to 100% (BMD), 85% to 92% (VITEK 2), 76% to 100% (gradient test), and 100% (disk diffusion).

3.2. Agreement of AST results for piperacillin/tazobactam using the MIC mode value of BMD and its susceptible or resistant category as the reference standard

Isolates harbouring $bla_{\text{TEM-1}}$ (26664 and 26273) (Table 2) with MICs around the breakpoint resulted in a % CA <80% for BMD when the mode value of BMD was used as the reference standard. The % EA for BMD was as low as 53% for isolate 26664. This issue was also observed in VITEK 2. VITEK 2 showed a very low % CA (<10%) in two isolates (26273 and 26239). In these isolates, VITEK 2 reported 'resistant' while other AST methods indicated 'susceptible'. The mode MIC value for isolate 26273 was 8 mg/L and for isolate 26239 was 4 mg/L.

The % EA of VITEK in most of the isolates was <90%, with the exception of isolates 24535 ('reverse' pattern and no β-lactamases) and 44782 (the piperacillin/tazobactam measurement failed the first time in VITEK 2) (Table 2).

3.3. Inter-reader reproducibility

The interrater reliability reading within ±1 doubling dilution was 99.3% for BMD and 96.8% for the MIC strip test.

4. Discussion

The AST methods tested here were shown to be robust, with % EA and % CA >90% for the challenging isolates included in

Table 1
Reproducibility of antimicrobial susceptibility tests for piperacillin/tazobactam in various challenging *Escherichia coli* isolates.

Isolate no.	Characteristic[*,†]	Broth microdilution				VITEK 2				MIC strip test				Disk diffusion	
		MIC mode value (S/R), mg/L	Mode value (%)[‡]	EA (%)	% CA[§] and errors	MIC mode value (S/R), mg/L	Mode value (%)[‡]	% EA	% CA[§] and errors	MIC mode value (S/R), mg/L	Mode value (%)[‡]	EA (%)	CA[§] and errors (%)	Mean (SD) zone diameter, (S/R), mm	CA (%)
26239	Reversed pattern and no β-lactamases, MICs of amoxicillin/clavulanic acid 4 mg/L, cefuroxime 4 mg/L, cefoxitin 4 mg/L, cefotaxime 0.5 mg/L, ceftazidime ≤0.125 mg/L, meropenem ≤0.125 mg/L, trimethoprim/sulfamethoxazole 1 mg/L, ciprofloxacin 0.25 mg/L	4 (S)	65	100	100	16 (R)	46	63	100	2 (S)	54	100	100	26 (0.5) (S)	100
24535	Reversed pattern and no β-lactamases, MICs of amoxicillin-clavulanic 4 mg/L, cefuroxime 8 mg/L, cefoxitin 4 mg/L, cefotaxime ≤0.25 mg/L, ceftazidime 0.25 mg/L, meropenem ≤0.125 mg/L, trimethoprim/sulfamethoxazole 1 mg/L, ciprofloxacin ≤0.25 mg/L	4 (S)	50	100	100	≤4 (S)	100	100	100	2 (S)	55	100	100	27 (0.7) (S)	100
26664	TEM-1, MICs of amoxicillin/clavulanic acid ≥32 mg/L, cefuroxime 2 mg/L, cefoxitin ≤4 mg/L, cefotaxime ≤4 mg/L, ceftazidime ≤1 mg/L, meropenem ≤0.125 mg/L, trimethoprim/sulfamethoxazole>8 mg/L, ciprofloxacin ≤0.25 mg/L	16 (R)	21	53	71 VME: 29%	≥128 (R)	65	65	85 VME: 15%	≥256 (R)	57	100	100	18 (2.5) (R)	100
26273	TEM-1, MICs of amoxicillin/clavulanic acid ≥32 mg/L, cefuroxime 4 mg/L, cefoxitin ≤4 mg/L, cefotaxime 0.5 mg/L, ceftazidime 0.5 mg/L, meropenem ≤0.125 mg/L, trimethoprim/sulfamethoxazole >8 mg/L, ciprofloxacin ≤0.25 mg/L	8 (S)	75	95	75 ME: 25%	16 (R)	76	100	92 VME: 8%	8 (S)	48	100	76 ME: 24%	21 (0.5) (S)	100
21530	AmpC, MICs of amoxicillin/clavulanic acid MIC ≥32 mg/L, cefuroxime >32 mg/L, cefoxitin >32 mg/L, cefotaxime 32 mg/L, ceftazidime >32 mg/L, meropenem ≤0.125 mg/L, trimethoprim/sulfamethoxazole >8 mg/L, ciprofloxacin ≤0.25 mg/L	>128 (R)	87	100	100	16 (R)	85	100	100	>256 (R)	100	100	100	13 (0.8) (R)	100
00150	OXA-1, MIC's of amoxicillin-clavulanic acid ≥32 mg/L, cefuroxime 16 mg/L, cefoxitin ≤4 mg/L, cefotaxime 0.125 mg/L, ceftazidime 0.25 mg/L, meropenem ≤0.125 mg/L, trimethoprim/ sulfamethoxazole 8 mg/L, ciprofloxacin ≤0.25 mg/L	>128 (R)	50	95	100	>128 (R)	95	100	100	>256 (R)	70	100	100	13 (0.5) (R)	100
44782	No β-lactamases. Measurement of piperacillin/tazobactam failed first time on VITEK 2. MICs for amoxicillin/clavulanic acid 8 mg/L, cefuroxime 16 mg/L, cefoxitin ≤4 mg/L, cefotaxime 0.125 mg/L, ceftazidime 0.25 mg/L, meropenem ≤0.125 mg/L, trimethoprim/ sulfamethoxazole 8 mg/L, ciprofloxacin ≤0.25 mg/	4 (S)	85	100	100	≤4 (S)	100	75	100	3 (S)	57	100	100	25 (0.5) (S)	100

For each isolate and each AST method, tests were repeated 20 times.

[*] 'Reverse' pattern implies susceptibility to amoxicillin/clavulanic acid but resistance to piperacillin/tazobactam during an initial AST using VITEK 2.

[†] The MIC of amoxicillin/clavulanic acid was repeated 10 times using VITEK 2 and all showed similar MICs.

[‡] The most frequent MIC value of each AST method.

[§] The same susceptible of resistant category using EUCAST breakpoint v. 14.0.CA, categorical agreement (defined as the same susceptible of resistant category as the category translated from the mode MIC of the specific AST method); EA, essential agreement (defined as the MIC within one doubling dilution as the MIC mode value); ME, major error; MIC, minimum inhibitory concentration; VME, very major error.[‡]Percentage of the similar MIC results as the most frequent MIC during repeated measures.

Table 2
Categorical agreement of antimicrobial susceptibility tests for piperacillin/tazobactam *Escherichia coli* isolates using mode value of broth microdilution as the reference standard.

Isolate number	Characteristic	Broth microdilution		VITEK 2		Disk diffusion		MIC strip test	
		CA	EA	CA	EA	CA	EA	CA	EA
26239	'Reverse' pattern* and no β-lactamases	100	100	0	0	100	NA	100	100
24535	'Reverse' pattern* and no β-lactamases	100	100	100	100	100	NA	100	100
26664	TEM-1	71.0	53	81.0	15	100	NA	100	0
26273	TEM-1	75.0	95	8.0	75	100	NA	76.0	100
21530	AmpC	100	100	85.0	0	100	NA	100	100
00150	OXA-1	100	100	100	95	100	NA	100	90
44782	Measurement of piperacillin/tazobactam failed first time on VITEK 2	100	100	100	100	100	NA	100	100

For each isolate, the test was repeated 20 times.
* Susceptible to amoxicillin/clavulanic acid but resistant to piperacillin/tazobactam during an initial AST using VITEK 2.CA, categorical agreement; EA, essential agreement; MIC, minimum inhibitory concentration; NA, not applicable.

this study aside from two *E. coli* isolates harbouring TEM β-lactamase (26664 and 26273). These isolates had MIC values of piperacillin/tazobactam close to the breakpoint. Our findings demonstrate that the reproducibility of tests on isolates with MICs around the breakpoint can be inconsistent when interpretative reading (i.e. susceptible or resistant categories) is used. This variability is perhaps simply the result of variability in measurement, which may become more pronounced for CLSI users with the recent lowering of breakpoints for piperacillin/tazobactam in Enterobacterales from $\leq$16 to $\leq$8 mg/L to align with the EUCAST breakpoint. This issue could be resolved by performing disk diffusion tests, which consistently show a similar CA with BMD and gradient test, and are robust (100% CA for all tested isolates). Disk diffusion may also help to resolve potential false resistance to piperacillin/tazobactam in *E. coli* in cases of 'reverse' direction resistance patterns on VITEK 2, which occurred in around 0.2% of VITEK 2 measurements in our laboratory.

Repeated MIC values as measured by BMD within one double dilution can be as low as 50%. This variability indicates that caution should be exercised in pharmacokinetic/pharmacodynamic studies when using a narrow range of piperacillin/tazobactam MIC values as a pharmacodynamic parameter, especially when the studies are focused on β-lactamase inhibitors.

In these challenging isolates, the VITEK 2 automated system tended to overestimate resistance, perhaps due to the use of advanced expert system algorithms. These algorithms compare MIC results against an extensive database to assess consistency. In routine practice, other AST methods such as the gradient test could be performed to confirm this result. Another observation regarding VITEK 2 in this study is that it often showed a low % EA when compared with BMD. This is perhaps due to inherent technical differences between AST methods, as often observed in studies comparing different AST methods, which could be specific to a certain AST method in combination with certain microorganisms [11–14]. When a laboratory needs to verify an AST method, we believe that BMD should be used as a reference method only when commercial BMD ASTs need to be verified. Unlike CLSI M52, during verification of ASTs ISO 20776-2:2021 does not use the terms CA, VME, and ME, as it argues that verification should measure test performance and not the results of interpretative reading using breakpoints. However, our results demonstrate that for certain isolates, inherent differences between AST methods can result in categorical agreement without essential agreement.

A strength of this study was that all tests were performed by one laboratory technician who was trained for this purpose to ensure reproducibility. Due to the paucity of data on this issue, we cannot compare the results of our study with others. A limitation of this study was that we did not further characterise bla_{TEM} to determine whether the problem with reproducibility was caused by the expression of inhibitor-resistant TEM.

In conclusion, this study provides insights into the reliability of AST results for piperacillin/tazobactam in challenging *E. coli* isolates. While the results indicate that most methods are generally reproducible, certain isolates may present inconsistent MIC results.

Declaration of competing interest

None declared.

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Ethical approval

This study used bacterial isolates; therefore, no ethical approval was required.

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Author contributions

E.Y. proposed the study idea. F.D. drafted the first report. All authors contributed to writing of the final report and approved the final version to be submitted.

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