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ORIGINAL ARTICLE

Multicenter interlaboratory study of routine systems for the susceptibility testing of temocillin using a challenge panel of multidrug-resistant strains

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Abstract

Accurate susceptibility result of temocillin (TMO) is important for treating infections caused by multidrug-resistant *Enterobacterales*. This multicenter study aimed to investigate the performance of routine temocillin testing assays against *Enterobacterales* challenging strains. Forty-seven selected clinical isolates were blindly analyzed by 12 Belgian laboratories using VITEK® 2 ($n=5$) and BD Phoenix™ ($n=3$) automated systems, ETEST® gradient strip ($n=3$), and disk (3 brands) diffusion method (DD; $n=6$) for temocillin susceptibility using standardized methodology. Results were interpreted using EUCAST 2023 criteria and compared to the broth microdilution (BMD; Sensititre™ panel) method used as gold standard. Methods' reproducibility was assessed by testing 3 reference strains in triplicate. A total of 702 organism-drug results were obtained against 33 TMO-susceptible and 14 TMO-resistant isolates. Excluding *Proteae* species (*P. mirabilis* and *M. morganii)*, the essential agreement rates were excellent (91.5–100%) for all MIC-based methods. The highest category agreement was achieved by ETEST® (97.5%) followed by VITEK® 2 (93.2%), disk diffusion (91.6%), and BD Phoenix™ (88.5%). BD Phoenix™ and paper disk diffusion overcalled resistance (11.5% and 6.8% of major discrepancies, respectively), while ROSCO tablets diffusion and VITEK® 2 generated higher very major discrepancies (7.1% and 4.2% respectively). Inter-assay reproducibility was unsatisfactory using recommended *E. coli* ATCC 25922 strain but was excellent with *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603 strains. This interlaboratory study suggests that routine testing methods provide accurate and reproducible TMO categorization results except for *Proteae* species.

Keywords Temocillin · Antimicrobial susceptibility testing · *Enterobacterales* · EUCAST Breakpoints

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Introduction and objectives

Multidrug resistance in Gram-negative rods represents a major public health issue impacting negatively on the outcome of infected patients. Infections by extended-spectrum β-lactamase (ESBL) or AmpC-producing *Enterobacterales* are often treated with carbapenems. However, the overuse of carbapenems could lead to the selection of resistance to these last-line treatments and a therapeutic dead-end. Therefore, the common practice in antibiotic stewardship aims to search for carbapenem-sparing regimen. Temocillin (TMO) is a narrow-spectrum carboxypenicillin with high stability to most β-lactamases produced by *Enterobacterales*, including ESBLs and AmpCs, and could serve as a useful alternative. Accurate antimicrobial susceptibility testing (AST) for temocillin is crucial to ensure clinical efficacy.

Until 2019, different AST interpretative breakpoints for temocillin were applied by clinical laboratories since they were proposed only based on the literature [1] or at a country level (BSAC, CASFM). In 2020, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) published breakpoints with major decisions: (1) all susceptible strains with a minimal inhibitory concentration (MIC) of ≤ 16 mg/L are categorized as "susceptible, increased exposure (I)" (no S result), requiring a high dosage regimen (2 g/8 h), whatever the MIC and the clinical setting; (2) there are only species-related breakpoints available for *Escherichia coli*, *Klebsiella* spp. (except *K. aerogenes*), and *Proteus mirabilis*; (3) indications for use are restricted to urinary tract infections (UTI) with comments on the distinction of uncomplicated UTI from urosepsis (www.eucast.org). The current EUCAST breakpoints are established based on wild-type distributions supplemented with limited pharmacokinetic/pharmacodynamic (PK/PD) data and scarce and sometimes contradictory clinical data [2–5]. Additionally, when setting breakpoints, the question on the accuracy of testing methods and reproducibility of results has also to be raised.

This study aimed to evaluate the analytical performances of different routine methods for the temocillin susceptibility testing used in different Belgian laboratories. It determines the accuracy and reproducibility of the methods, when performed on a collection of *Enterobacterales* isolates with variable level of susceptibility to temocillin and different resistance mechanisms to β-lactams.

Materials and methods

The study panel included 47 previously phenotypically and/or genotypically characterized non-duplicate clinical isolates belonging to 10 *Enterobacterales* species are summarized in Table 1 and beta-lactam resistance mechanisms are detailed in Supplementary data S1. Fourteen (including seven from a previous study [6]) temocillin-resistant (TMO-R) and 33 temocillin susceptible "increased exposure" (TMO-I) strains, showing a wide range of inhibition diameters, were selected, based on disk diffusion susceptibility according to the EUCAST 2023 guidelines. Such selection allowed testing of various levels of temocillin resistance, including those close to the EUCAST breakpoint (I ≥ 17 mm), thereby challenging different routine AST methods used by 12 Belgian laboratories.

Among laboratories performing automated susceptibility method (AUST), three used BD Phoenix™ with NMIC-408 panel (Becton–Dickinson, Sparks, USA), while five used VITEK® 2 with AST-N366 card (bioMérieux, Marcy

Table 1 Characteristics of clinical isolates ($n = 47$) tested in the study

Species	Mechanisms of resistance to beta-lactams	TMO susceptible ($n = 33$)	TMO resistant ($n = 14$)
E. coli ($n = 11$)	WT ($n = 1$), ESBL ($n = 6$), carbapenemases ($n = 3$), pAmpC ($n = 1$), hpAmpC ($n = 1$)	8	3
K. pneumoniae ($n = 15$)	WT ($n = 2$), ESBL ($n = 6$), carbapenemases ($n = 5$), pAmpC ($n = 3$)	8	7
K. oxytoca ($n = 4$)	WT ($n = 1$), ESBL ($n = 2$), carbapenemase ($n = 1$)	4	0
K. aerogenes ($n = 2$)	WT ($n = 1$), hpAmpC ($n = 1$)	2	0
C. koseri ($n = 2$)	WT ($n = 1$), carbapenemase ($n = 1$)	2	0
C. freundii ($n = 3$)	WT ($n = 1$), ESBL ($n = 1$), hpAmpC ($n = 1$)	3	0
E. cloacae complex ($n = 4$)	WT ($n = 1$), hpAmpC ($n = 2$), ESBL ($n = 1$), carbapenemase ($n = 1$)	2	2
S. marcescens ($n = 2$)	WT ($n = 1$), hpAmpC ($n = 1$)	1	1
P. mirabilis ($n = 2$)	ESBL ($n = 1$), carbapenemase ($n = 1$)	1	1
M. morganii ($n = 2$)	ESBL ($n = 2$)	2	0

Caption: *WT*, wild type; *ESBL*, extended-spectrum beta-lactamase; *pAmpC*, plasmidic AmpC cephalosporinase; *hpAmpC*, hyperproduced chromosomal AmpC cephalosporinase

l'Etoile, France). All test cards used by each laboratory originated from the same batch.

Three laboratories used gradient strip diffusion with ETEST® temocillin (bioMérieux, Marcy l'Etoile, France), while six performed diffusion of temocillin 30-µg disk of three different brands: BioRad ($n=3$), Hercules, CA, USA, Becton–Dickinson ($n=2$), Franklin Lakes, NJ, USA and ROSCO Diagnostics ($n=1$), Taastrup, Denmark. The manufacturer of the Mueller–Hinton agar plates used for each diffusion method is detailed in Supplementary data S2.

Reproducibility was evaluated by testing three reference strains in triplicate for all methods: *E. coli* ATCC 25922 [7], *E. coli* ATCC 35218 [8], and *K. pneumoniae* ATCC 700603. Acceptable ranges for the MIC and for the inhibition zone diameter (IZD) were as follows: *E. coli* ATCC 25922 (MIC: 8–32 mg/L IZD: 16–22 mm) [7], *E. coli* ATCC 35218 (MIC: 2–8 mg/dL; IZD: 19–28 mm) [9], and *K. pneumoniae* ATCC 700603 (MIC: 8–16 mg/dL; IZD: 14–20 mm based on repeated weekly testing for 6 months at the NRC).

All strains were dispatched to the different participating laboratories and testing was carried out on freshly prepared overnight subcultures on non-selective agar plates. Each laboratory verified isolates bacterial identification of the isolates using MALDI-TOF MS (Bruker, Massachusetts, USA) or Vitek MS (bioMérieux, Marcy l'Etoile, France). AST was performed once on 47 clinical collection strains and in triplicate on the 3 reference strains in compliance with EUCAST methodology including disk diffusion reading instructions [9]. Any invalid result for temocillin was retested using the same method. Reference MIC and category results for TMO were defined by broth microdilution (BMD) using customized Sensititre™ panels (BEGN5A, Thermo Fisher Scientific, Waltham, MA, USA) at the National Reference Center for Antibiotic-Resistant Gram-Negative Bacilli (NRC). Readers were blinded to the temocillin results of the reference method and to the microbiological characteristics (beta-lactam resistance mechanisms) of the tested strains.

Recorded raw results values were centralized and interpreted by the NRC according to the EUCAST 2023 clinical breakpoints for temocillin [9]. The TMO MIC and category results obtained by different methods were compared to the BMD results. Categorical agreement (CA: agreement of category results), essential agreement (EA: MICs within ± 1 dilution of reference MICs, adapted to the range of the tested dilutions by excluding all extreme values of ≤ X and > Y mg/L), absolute agreement (AA: identical MIC values), very major discrepancy (VMD: false TMO-I result), and major discrepancy (MD: false TMO-R result) rates were calculated for each method compared to the reference BMD. All methods were evaluated using ISO Standard 20776–2 criteria (EA and CA > 90%, VMD < 3%).

Results

Reproducibility on reference strains

Fifty-four TMO results per strain were obtained for reproducibility testing (Table 2). Results for the recommended *E. coli* ATCC 25922 showed a wider range of MIC (5 twofold dilutions) and IZD (8 mm) including more than one out-of-range result for BD Phoenix™ and Rosco. Only BMD, ETEST®, and DD using BD disk methods showed perfect reproducibility within acceptable results range. On the other hand, *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603 yielded a narrower results range (of 3 MIC dilutions and of 6 mm IZD) with only one out-of-range result for each strain.

Method comparison on clinical collection strains

In total, 700 organism-drug results were acquired. All agreement and discrepancy rate results are detailed in Table 3. No invalid results were observed.

MIC-based methods

A total of 221, 128, and 124 organism-drug results were obtained to calculate categorization performance (CA, VMD, MD) for VITEK® 2, BD Phoenix™, and ETEST®, respectively. Due to truncations in the concentration range of the evaluated method and/or of the reference method, the numbers of evaluable organism-drug results were lower for the calculation of EA and AA (75, 70, and 85 for VITEK® 2, BD Phoenix™, and ETEST® methods, respectively).

For all 47 isolates, the ETEST® method demonstrated a higher CA than the AUST methods, even though the AUST methods achieved a better EA than the ETEST® method. This method resulted in 4% of VMD and 0.8% of ME for all 47 isolates. We observed a higher rate of VMD (8.3%) for species other than *E. coli* and *K. pneumoniae*. Compared to other species, higher CA using MIC methods was reached with *K. pneumoniae*.

Regarding the BD Phoenix™ method, we observed a high rate of MD ranging from 11.5 to 20.7% among *Enterobacterales* except for *K. pneumoniae* isolates where no MD was observed. The VITEK® 2 method yielded the highest rate of VMD, ranging from 3.1 to 9.4% among *Enterobacterales*. No interpretative result was provided by BD Phoenix™ for *M. morganii* isolates.

Better performance for MIC-based methods was achieved when results for *Proteae* isolates (*P. mirabilis*

Table 2 Reproducibility (%) of the temocillin testing method on reference strains

TMO AST	Acceptable range		MIC method (rates of values within acceptable range)				DD method (rates of values within acceptable range)			
QC strain (n = number of results per strain)	MIC (mg/L)	IZD (mm)	Observed range (mg/L)	Sensititre™ BMD (n = 3)*	ETEST® (n = 9)	BD Phoenix™ (n = 9)	VITEK® 2 (n = 15)	Observed range (mm)	BD (n = 6)	BioRad (n = 9)	ROSCO (n = 3)
E. coli ATCC 25922 [7]	8–32	16–22	4–64	100%	100%	78%	87%	17–24	100%	89%	33%
E. coli ATCC 35218 [8]	2–8	19–28	4–16	100%	100%	89%	100%	22–27	100%	100%	100%
K. pneumoniae ATCC 700603*	8–16	14–20	4–16	100%	100%	100%	93%	14–20	100%	100%	100%

Caption: *TMO*, temocillin; *AST*, antimicrobial susceptibility testing; *BMD*, broth microdilution; *DD*, disk diffusion; *IZD*, inhibition zone diameter; *MIC*, minimal inhibitory concentration; *QC*, Quality control

*Data from Belgian National Refence for Multi Resistant Center for Bacilli Gram Negative Bacilli

and *M. morganii*) were excluded from the analysis, resulting in the increase of the CA and the EA, and lowering the VMD for all three methods (see Table 3). However, the AA was poor for all methods (49.3% to 51.8%).

Disk diffusion method

Of the 274 organism-drug combinations obtained with all 47 tested isolates, disk diffusion methods globally achieved 90.9% of CA, 2.9% of VMD and 6.2% of MD, 91.6% of CA, 1.6% of VMD, and 6.8% of MD were obtained when *Proteae* strains were excluded.

The ROSCO tablet disk method had the highest rate of VMD compared to the other DD methods, ranging from 5.9 to 9.5% among subgroups of *Enterobacterales*.

Paper disk (BioRad and BD) methods gave general CA rates of > 90%, but high MD rates of 6.9 to 7.8%. *Escherichia coli* showed the highest MD (12.1 to 14.2%) and the lowest CA (84.8 to 85.7%). Most of the VMD originated from one strain each of OXA-48 carbapenemase-producing-*P. mirabilis* (strain TEMO-S38) and of OXA-48 carbapenemase-producing-*E. coli* (strain TEMO-S09) while the predominant source of ME was generated by one *E. coli* strain (strain TEMO-S06) with MIC close to the clinical breakpoint (MIC = 16 mg/L) (Table 4).

Discussion

Clinical breakpoints for susceptibility testing of temocillin were released by EUCAST interpretation guidelines in 2020. The updated recommendations allow only "I" (susceptible to increased exposure) results for non-TMO-R strains, requiring administration of a high temocillin dosing regimen (2 g/8 h) for infections originating from the urinary tract. However, several groups have demonstrated that temocillin administered at 2 g/12 h can be effective in the treatment of uncomplicated urinary tract infections (uUTI) and of complicated urinary tract infections (cUTI), with bacteriemia caused by *Enterobacterales* strains with a maximal MIC of 8 mg/L, irrespective of the species involved [2, 10]. The choice to administer standard doses (2 g/12 h) versus high doses (2 g/8 h) of temocillin remains controversial with potential impacts on financial and stewardship considerations, thereby highlighting the essential need for a reproducible and reliable method for temocillin laboratory testing. A previous study showed that a breakpoint of 8 mg/L and a zone diameter of 22 mm were most accurate to determine temocillin susceptibility and > 32 mg/L and 12 mm were accurate to determine temocillin resistance for all isolates [11]. Additionally, in Belgium, despite extensive clinical usage for more than three decades, temocillin has retained high and constant in vitro activity against *E. coli* and *K. pneumoniae* showing 98.1%

Table 3 Performance of MIC-based temocillin testing methods

TMO MIC-based method (n labs)	Etest (n=3)					BD Phoenix™ (n=3)					VITEK® 2 (n=5)				
Species (total n isolates)	CA(%) %[95CI]	VMD(%) %[95CI]	MD(%) %[95CI]	AA(%) %[95CI]	EA(%) %[95CI]	CA(%) %[95CI]	VMD(%) %[95CI]	MD(%) %[95CI]	AA(%) %[95CI]	EA(%) %[95CI]	CA(%) %[95CI]	VMD(%) %[95CI]	MD(%) %[95CI]	AA(%) %[95CI]	EA(%) %[95CI]
All (n=47)	95,2 [89,8-97,7]	4,0 [1,7-9,0]	0,8 [0,1-4,4]	51,8 [41,2-62,1]	88,2 [78,3-92,6]	85,4 [78,3-90,4]	2,3 [0,8-19,1]	12,3 [7,7-19,1]	54,3 [42,7-65,4]	93,5 [82,5-9,8]	87,3 [82,3-91,1]	7,2 [4,5-11,4]	5,4 [3,1-9,2]	49,3 [38,3-60,4]	90,6 [82,0-95,4]
All except PM/MM (n=43)	97,5 [92,8-99,1]	1,7 [0,4-5,9]	0,8 [0,1-4,6]	53,7 [42,9-64,0]	91,5 [83,3-95,8]	88,5 [81,6-93,0]	0,0 [0,0-3,0]	11,5 [7,6-19,3]	57,6 [45,6-68,7]	100 [91,8-100]	93,2 [88,7-95,6]	4,2 [2,1-11,7]	2,6 [1,1-5,9]	51,6 [39,4-63,6]	96,7 [88,8-99,1]
EC (n=11)	100 [89-100]	0,0 [0,0-11,0]	0,0 [0,0-11,0]	61,9 [40,8-70,2]	100 [84,5-100]	87,5 [71,9-95,0]	0,0 [0-10,7]	12,5 [4,9-28,1]	52,4 [49,7-71,6]	100 [74,1-100]	81,1 [68,6-89,4]	9,4 [4,0-20,2]	9,4 [4,0-20,2]	22,2 [9,0-45,2]	100 [82,4-100]
KP (n=15)	100 [89,0-100]	0,0 [0-10,4]	0,0 [0-10,4]	44,0 [26,6-62,9]	88,0 [70,0-95,8]	100 [91,2-100]	0,0 [0-8,8]	0,0 [0-8,8]	70,0 [48,1-85,4]	100 [83,9-100]	96,9 [89,4-99,1]	3,1 [0,8-10,5]	0,0 [0-5,6]	63,6 [42,9-80,3]	90,9 [72,2-97,4]
Non-EC/KP (n=21)	90 [79,8-95,3]	8,3 [3,0-18,1]	1,7 [0,3-8,9]	55,6 [39,6-70,5]	92,6 [83,0-97,2]	74,1 [61,2-83,6]	5,2 [1,8-14,1]	20,7 [12,2-62,4]	44,8 [28,4-62,4]	82,4 [91,8-100]	84,5 [73,2-90,2]	8,7 [4,6-15,8]	6,8 [3,3-13,3]	54,3 [38,2-69,5]	85,7 [70,6-93,7]
Non-EC/KP/PM/MM (n=17)	94,4 [84,8-98,0]	3,7 [1,0-9,8]	1,9 [0,3-9]	55,5 [39,5-70,4]	92,5 [82,4-97,1]	80 [67,0-88,8]	0 ,0 [0-7,1]	20 [11,2-33,0]	52,0 [33,5-70,0]	100 [78,5-100]	95,2 [88,2-98,1]	4,8 [1,8-11,7]	0,0 [0-4,4]	59,2 [40,7-75,4]	100 [95,6-100]

Caption: EC: *E. coli*; KP: *K. pneumoniae*; MM: *M. morganii*; PM: *P.mirabilis*; %[95CI]: Confidence Interval of 95%; TMO: temocillin; AA: Absolute agreement; CA: Categorical agreement; MD: Major discrepancy; VMD: Very Major Discrepancy;

Interpretation criteria:

VMD/MD (%)	0-3%	VMD/MD (%)	3-5%	VMD/MD (%)	>5%
CA/EA (%)	<80%	CA/EA (%)	80-90%	CA/EA (%)	90-100%

Table 4 Performance of temocillin disk diffusion methods

TMO disk diffusion (n labs)	Total disks (n=6)			BioRad (n=3)			BD (n=2)			Rosco (n=1)		
Species (total n isolates)	CA(%) %[95CI]	VMD(%) %[95CI]	MD(%) %[95CI]	CA(%) %[95CI]	VMD(%) %[95CI]	MD(%) %[95CI]	CA(%) %[95CI]	VMD(%) %[95CI]	MD(%) %[95CI]	CA (%) %[95CI]	VMD(%) %[95CI]	MD (%) %[95CI]
All (n=47)	90,9 [86,9-93,7]	2,9 [1,5-5,6]	6,2 [3,9-9,7]	90,1 [84,0-93,4]	2,1 [0,7-6,1]	7,8 [4,4-13,4]	92,0 [84,3-96,0]	1,1 [0,2-6,2]	6,9 [3,2-14,2]	91,3 [79,7-96,6]	8,7 [3,4-20,3]	0,0 [0,0-7,7]
All -PM/MM (n=43)	91,6 [87,5-94,4]	1,6 [0,6-4,0]	6,8 [4,3-10,6]	90,7 [84,4-94,6]	0,8 [0,1-4,3]	8,5 [4,8-14,6]	92,4 [84,4-96,5]	0,0 [0-10,6]	7,6 [3,5-15,6]	92,9 [78,4-96,3]	7,1 [2,4-18,6]	0,0 [0,0-8,2]
EC (n=11)	86,2 [75,7-92,5]	3,1 [0,8-10,6]	10,7 [5,3-20,6]	84,8 [69,1-93,3]	3,0 [0,5-15,3]	12,1 [4,8-27,3]	85,7 [65,3-95,0]	0 ,0 [0-15,4]	14,2 [4,9 -34,6]	90,9 [62,2-98,4]	9,0 [1,6-37,6]	0,0 [0-25,8]
KP (n=15)	92,9 [85,2-96,7]	1,1 [0,2-6,4]	6,0 [2,5-13,1]	93,3 [82,1-97,7]	0,0 [0-7,8]	6,7 [2,9-17,9]	92,0 [75,0-97,7]	0 ,0 [0-13,3]	8,0 [2,2-27,9]	92,8 [68,5-98,7]	7,1 [1,2-31,5]	0,0 [0-21,5]
Non-EC/KP (n=21)	92,0 [85,9-95,6]	4,0 [1,2-7,9]	4,0 [1,2- 7,9]	90,5 [80,7-95,6]	3,2 [0,8-10,8]	6,3 [2,5-15,2]	95,1 [83,9-98,6]	2,4 [0,4-12,6]	2,4 [0,4-12,6]	90,5 [71,1-97,3]	9,5 [2,6-28,9]	0,0 [0,0-15,5]
Non-EC/KP/PM/MM (n=17)	94,0 [87,6-97,2]	1,0 [0,2-5,4]	5,0 [2,1-11,1]	92,2 [81,5-96,7]	0,0 [0-7,0]	7,8 [3,1-,18,5]	97,0 [84,7-99,4]	0,0 [0-10,4]	3,0 [0,5-15,3]	94,1 [73,0-98,9]	5,9 [1,0-26,7]	0,0 [0,18,4]

Caption: EC: *E. coli*; KP: *K. pneumoniae*; MM: *M. morganii*; PM: *P.mirabilis*; %[95CI]: Confidence Interval of 95%; TMO: temocillin;AA: Absolute agreement; CA: Categorical agreement; MD: Major Discrepancy; VMD: Very Major Discrepancy

Interpretation criteria:

VMD/MD (%)	0-3%	VMD/MD (%)	3-5%	VMD/MD (%)	>5%
CA (%)	<80%	CA (%)	80-90%	CA (%)	90-100%

and 97.8% of susceptibility, respectively, according to the data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) [12].

While being the recommended strain for quality control of temocillin AST methods [7], perfect accuracy rates for *E. coli* ATCC 25922 were only achieved with our reference method (Sensititre™ BMD) and with ETEST®. On the other hand, *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603 strains gave more reproducible results with fewer variations (smaller range) of MIC/IZD values between methods. The data presented here suggest that the different AST methods can be considered reproducible and that *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603 can serve as additional and more reliable QC strains than *E. coli* ATCC 25922, which may not be the best candidate for evaluating the reproducibility of temocillin (potentially unstable expression of resistance and overlapping with clinical breakpoints). Our observations were in line with one previous report [8], and we support further validation studies for including these strains into routine QC of TMO testing.

With a collection of 47 clinical strains, we evaluated the performance for the categorization (I/R) of TMO results and the accuracy of MIC provided by routine methods. The ETEST® method demonstrated a satisfactory CA (95.2%), but there are questions about the performance of other methodologies. Neither AUST methods nor disk diffusion met the CA target of over

90% and the VMD target of less than 3%. However, it should be noted that our study included a selected panel of challenging strains expressing varied levels of resistance to temocillin, which may slightly impair the performance indicators. A study by Alexandre et al. showed excellent performance of Vitek2, ETEST, and disk diffusion methods showing no very major error and < 5% of major error rates; however, the routine methods were compared to the agar dilution as reference method and tested on consecutive clinical urinary *Enterobacterales* isolates that were all temocillin susceptible at increased exposure (only 3/762 isolates had MIC between 8 and 16 mg/L) [13].

Interestingly, CA improved to over 90% for all methods except BD Phoenix™ when results from 4 *Proteae* isolates (one OXA-48 carbapenemase- and one OXA-1 penicillinase-producing *P. mirabilis*; one OXA-1 penicillinase-producing and one CTX-M group 1 ESBL-producing *M. morganii*) were excluded from the analysis, suggesting that the methods employed are generally reliable for non-*Proteae* and, at the same time, questioning the validity of our reference method (Sensititre™ BMD) for the testing of species belonging to *Proteae* (*Proteus* spp., *Providencia* spp., and *M. morganii*). A recent warning document released by Thermo Fisher Scientific (Thermo Scientific Sensititre Gram Negative AST Sensititre plate Technical Bulletin 2023) alerted potential inaccuracy of susceptibility results by Sensititre™ panels for carbapenems, cefepime, piperacillin/tazobactam, and aztreonam against *Proteae*, but temocillin was not addressed. This omission could result from the non-evaluation of the agent by the manufacturer, thus the performance of temocillin testing by Sensititre™ for this *Enterobacterales* group.

Regarding the ability to determine MIC value, we found a rather poor absolute agreement (identical MIC value) of only around 50% for all evaluated MIC methods. This uncertainty regarding an exact MIC value further underpins the discussion on the usage of high versus standard doses of antibiotics, especially when the obtained MIC obtained is between 8 and 16 mg/L.

The BD Phoenix™ method appeared to overestimate the resistance of *Enterobacterales* to temocillin (ME = 12.3%) except for *K. pneumoniae* isolates, for which no false resistance was detected. Our observations were similar to those obtained by two previous studies [14, 15]. Regarding the performance for *Proteae*, only one study assessed the susceptibility testing of multidrug-resistant ESBL-producing *P. mirabilis* that showed EA and CA > 95% for the Phoenix System compared to the E-test method considered as reference method [16]. However, temocillin testing was not evaluated in this study. On the other hand, VITEK® 2 produced a high rate of false susceptibility, particularly for strains with MIC values close to the breakpoint (8 and 16 mg/L). Therefore, we recommend confirming these MIC values by a BMD method.

Based on the excellent EA (within ± one doubling dilution) of most MIC-based methods, our data could potentially contribute to refine the susceptibility breakpoints proposed by EUCAST, by introducing an "S" category (with a "S" breakpoint set lower than 16 mg/L) specifically for the treatment of uncomplicated UTI caused by *Enterobacterales* strains, although with the risk of splitting the wild-type distribution among some species. Such approach was taken in the recent guidelines of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CASFM) which introduced in June 2023 a "S" category for strains with MIC ≤ 8 mg/L allowing the use of standard dose in case of uncomplicated UTI [17] supported by clinical studies [2, 18].

Regarding the disk diffusion methods, paper disk diffusion showed performant and reliable categorization, with high agreement with the reference method for strains with IZD ≥ 17 mm (corresponding to the TMO-I category) or < 12-mm diameter. A poorer agreement was observed for results of isolates falling within the IZD range of 13 to 16 mm for which false resistance was observed for half (11/22) of the strains. Therefore, a secondary method might be needed to confirm these R results (IZD between 13 and 16 mm) to avoid missing the opportunity for clinical use. This finding deserves additional studies by increasing the number of strains tested to define or even reduce such zone as a potential area of technical uncertainty (ATU) according to EUCAST, which can further improve the accuracy of the TMO disk diffusion method. Of note, the high rate of VMD generated by ROSCO tablet diffusion raised concern about its validity, but the limited dataset, generated by one single laboratory, withheld from drawing definitive conclusions.

A major strength of our multicenter study lies in the standardized methodology employed by different participating centers. Our study used the same batch of bacterial strains and testing materials (same-lot ETEST® strips and AUST panel cards) distributed centrally except for disk diffusion materials. This harmonized approach has contributed to results' reliability and validity. However, our study had some limitations. First, only a small number of selected strains was evaluated and limited results per method was available, particularly in species other than *E. coli* and *K. pneumoniae*. Then, the performance of the evaluated methods using selected challenge strains (with susceptibility close to the breakpoint) for our study might be lower than in a routine setting testing random isolates. Finally, the validity of the Sensititre™ broth microdilution (BMD) method as the reference standard could be questioned based on the lack of poor reproducibility and high variation of results specifically for the testing of *Proteae*.

Conclusion

Our findings indicate that commercial routine methods used in clinical laboratories provide accurate and reproducible temocillin susceptibility results, although confirmatory test

might be necessary for results close to the clinical breakpoint. The inclusion of reference strains other than EC25922 displaying fewer variable results for the quality control of temocillin testing should also be considered.

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Author contribution All authors contributed to the study conception and design. Management and logistics of materials and strains' preparation were performed by Kris Vernelen and Pieter-Jan Ceyssens. Data collection and analysis were performed by Corentin Deckers and Te-Din Huang. The manuscript was written by Corentin Deckers and reviewed by Te-Din Huang. All authors commented on previous versions of the manuscript and approved the final version of the manuscript.

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Data availability The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Declarations

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