Categorization of temocillin susceptibility using the disc diffusion method: Susceptible, Intermediate, Resistant or Undetermined?

Amandine HENRY (amandine.henry@free.fr)
Hopital Andre Mignot https://orcid.org/0000-0002-8935-8185
Anthony Michaud
Centre Hospitalier Universitaire de Poitiers
Nicolas Degand
Centre Hospitalier Universitaire de Nice
Emmanuelle Bille
Hopital universitaire Necker-Enfants malades Maladies rares et chroniques
Damien Fournier
Centre Hospitalier Universitaire de Besancon
Agathe Capitaine
Centre Hospitalier Universitaire de Caen
Cecile Le Brun
Centre Hospitalier Regional Universitaire de Tours
Julie Lourtet-Hascoët
Groupe hospitalier Paris Saint-Joseph
Thomas Guillard
Centre Hospitalier Universitaire de Reims
Stéphane Corvec
Centre Hospitalier Universitaire de Nantes
Chloé Plouzeau-Jayle
Centre Hospitalier Universitaire de Poitiers
Elena Guillotel
Centre Hospitalier Universitaire de Rennes
Eric Farfour
Hopital Foch

Short report

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Abstract

Background: Since the pandemic of ESBL (extended-spectrum beta-lactamases) and the emergence of carbapenemase-producing *Enterobacterales* there is a renewed interest in temocillin. However, as the molecule was little used, except in Belgium and UK, there are few guidelines for its antibiotic susceptibility testing (AST). We aim to assess the accuracy of the disc diffusion method (DDM) for temocillin susceptibility testing.

Methods: Eight hundred eighty-eight *Enterobacterales* clinical strains of which 61.7% were resistant to 3 rd generation cephalosporin (3GC-R) were included. AST was performed using DDM in comparison to gradient strip tests (GST) and interpreted using the diameter breakpoint of 20mm and MIC breakpoint of 8 mg/L for DDM and GST as recommended by EUCAST-CASFM recommandations.

Results: At breakpoint of 8mg/L, temocillin rates of susceptibility were 76.9%, 95.6% and 67.7% for overall, 3GC-S and 3GC-R strains respectively. Ninety-four (10.6%) discrepancies were noticed including 68 (72.3%) major errors and 26 (27.6%) very major errors. Sixty-eight (72.3%) of all errors correspond to inhibition diameters comprised between 17 and 23 mm. The presence of colonies within the inhibition diameter was noticed for 32 (5.3%) strains susceptible to temocillin.

Conclusions: DDM lacks accuracy for strains displaying borderline inhibition zone diameter. We suggest applying the concept of area of technical uncertainty (ATU) for standard DDM. The strains displaying a diameter within the ATU should be tested by another method. The significance of colonies within the inhibition zone diameter should be explored.

Background

As a consequence of the pandemic of Extended Spectrum Beta-lactamase (ESBL), and in order to prevent broad-spectrum antibiotics use, there is a recently renewed in temocillin interest. Indeed, the molecule structure presents a 6-α-methoxy group conferring intrinsic stability against most beta-lactamases such as penicillinase, extended-spectrum beta-lactamase (ESBL), AmpC, and KPC carbapenemase [1]. Temocillin rate of resistance was assessed ranging from less than 5% to up to 40% in strain susceptible (3GC-S) and resistant (3GC-R) to 3rd generation cephalosporin respectively [2,3]. Nevertheless, as the molecule was little used, except in few countries such as Belgium and UK, there are few guidelines for temocillin susceptibility testing method and interpretation. Clinical laboratories mainly use minimal inhibitory concentration (MIC) determination by gradient strip test or microdilution and qualitative categorization using the disc diffusion methods (DDM). Previous reports have shown robust reliability of the DDM for *in vitro* susceptibility testing, but some limits were highlighted regarding manufacturer or bacterial species [2,4–6]. In the present study, we aim to assess the reliability of the DDM in comparison to MIC assessed by a gradient strip test method. This multicenter project (GMC12b) was supported by the GMC study group, an association of 30 microbiologists involved in clinical research.

Methods

Eleven clinical laboratories participated in the study. Each center was invited to include at least 50 *Enterobacterales* strains of which at least 60% were 3GC-R. MICs were determinated by ETEST® (bioMérieux, Marcy l'Etoile, France) as recommended by the manufacturer. DDM was performed as previously described [7]. Briefly, a Mueller Hinton agar was inoculated using a 0.5 Mc Farland calibrated suspension and a 30µg disc of temocillin was applied within at most 30 min. The plate was then incubated as recommended [7]. The suppliers of temocillin discs were Biorad (Hercules, California, United States) and I2a (Montpellier, France) for 657 (74.0%) strains (4 centers) and 231 (26.0%) strains (8 centers) respectively. One center performed DDM using first BioRad and then I2a reagent.

According to French guidelines, susceptibility to temocillin was interpreted using the breakpoint of 8 mg/L and 20 mm for ETEST® and DDM methods respectively [7]. Mechanisms of 3CG resistance (i.e. ESBL and cephalosporinase overproducing (COPE) isolates) were distinguished using a phenotypic-based approach [7]. Considering the ETEST® as the reference method, DDM results were categorized as either concordant, major error (susceptible by ETEST® but resistant by DDM) or very major error (resistant by ETEST® but susceptible by DDM).

Statistical analysis was performed using the SSPS software and the Chi-square tests.

Results

Clinical isolates and temocillin susceptibility

A total of 888 clinical isolates were included. There were mainly collected from blood cultures (72%), urines (16.8%), suppuration (4.8%), respiratory samples (2.4%) or other clinical samples (4.0%). *Escherichia coli* (51.7%) was the most frequent species, followed by *Klebsiella pneumoniae* (15.1%), *Enterobacter cloacae* complex (14.2%), *K. aerogenes* (4.3%) and *K. oxytoca* (2.0%) (Table 1). Other *Enterobacterales* species account for 12.7%. Overall, 548 (61.7%) strains were 3GC-R of which 318 (58.1%) were ESBL-E.

At breakpoint of 8mg/L, the temocillin susceptibility rate were 76.9%, 95.6% and 67.7% for overall, 3GC-S, 3GC-R strains respectively. MIC_{50} were 4 mg/L, and 8 mg/L for 3GC-S and 3GC-R strains. MIC_{90} were respectively 8 mg/L and 24 mg/L (figure 1, table 2). The temocillin susceptibility rate was significantly different according to the C3G-R mechanism : 71,6% for ESBL-E and 60.1% for COPE (*P<0.05*).

Temocillin susceptibility rates were significantly higher using ETEST® than DDM (76.9% vs 72.2%, *P<0.05*).

Accuracy of temocillin susceptibility testing by DDM

Comparison of MICs and inhibition diameters revealed 94 (10.6%) discrepancies including 68 (72.3%) major errors and 26 (27.6%) very major errors (Table 2). This rate of discrepancies was significantly higher for 3GC-S than 3GC-R strains (5.2% versus 14.1%, *P<0.01*).

Seventy-five (79.8%) of all errors were reported for inhibition diameters comprised between 17 and 23 mm. Among these, 53 (70,7%) and 22 (29,3%) were major and very major respectively (Figure 2). Among the 609 strains displaying a susceptible inhibition diameter (≥20mm), the presence of colonies within the inhibition diameter was noticed for 32 (5.3%) strains. All except two strains were reported as susceptible by ETEST®. MIC value was 12mg/L for these 2 strains.

The overall error rate was 8.7% and 16% using BioRad and I2A disks respectively (*P<0.01*). Using I2a reagent, most errors were ME (91.9%), while they accounted for 40.4% using BioRad reagents. For both suppliers, most errors occurred for an inhibition diameter between 17 and 23 mm (86% and 70.3% for BioRad and I2a respectively). Regarding these strains, very major errors were significantly higher using BioRad discs (3,8% versus 42,9%, *P<0,05*) (Table 2).

Discussion And Conclusion

Our results strongly suggest that DDM methods present several limits for assessing temocillin susceptibility. Most errors were observed for strains displaying borderline inhibition zone diameter of 17 to 23 mm suggesting these strains should be tested by another method. We used Etest as a reference for temocillin susceptibility testing as it previsouly showed good correlation with broth microdilution [4,8].

At breakpoint of 8mg/L, the rate of discrepant using the DDM was previously assessed bellow 5% by most report [2,5,6,8]. Rodriguez-Villalobos *et al.* described a higher overall error rate of 15% [4]. This latter study mainly included 3GC-R strains in contrast to reports describing a good accuracy of the DDM. Alexandre *et al.* previously reported a good accuracy of the routine methods for temocillin AST including DDM [2]. However, they focused on *Enterobacterales* isolates collected from community-acquired urinary tract infections. Most of these strains were susceptible to 3GC and temocillin (with a temocillin susceptibility rate of 99.6%). In the present study, the strains were isolated from both community and health-care-associated infections and 61.7% were 3GC-R. As previously reported, despite the temocillin stability against most beta-lactamase, 3GC-R strains display higher MICs to temocillin than 3GC-S strains [9]. Moreover, the modal distribution of temocillin MICs in *Enterobacterales* is close to the breakpoint of 8 mg/L [10,11]. These facts would probably explain why i) most errors occur for borderline inhibition diameter of 17-23 mm and, ii) 3GC-R strains are more likely incorrectly categorized using the DDM, iii) discrepant results were previously reported for some species expressing an *AmpC* such as *Serratia spp* [5].

Recently, EUCAST guidelines has introduced the concept of an area of technical uncertainty (ATU): “ATU is an area where the interpretation is uncertain, due to the poorer separation between susceptible and resistant isolates” [12]. We suggest introducing this concept for temocillin susceptibility testing using the

DDM. Consequently, the isolates displaying an inhibition zone diameter ranging from 17 to 23 mm, should be tested using another method: i.e. microdilution, gradient strip test.

DDM is particularly challenging due to the presence of colonies within the inhibition zones for some bacterial/antibiotics couple: i.e. fosfomycin and *Enterobacterales*. This phenomenon was observed for about 10% of all temocillin susceptible strains included here. They were not taking into account as they were not recovered by the gradient strip test. However, this phenomenon needs to be explored to find out if these colonies are part of a mutant resistant subpopulation or if they are artifact/mutant generation due to DDM.

DDM accuracy also seems to depend on supplier reagent. The overall rate of error is higher using I2a discs which are likely to provide ME (i.e. resistant by DDM while susceptible by ETEST®). Therefore, using this reagent, one could consider controlling temocillin susceptibility only for strains displaying an inhibition diameter comprise between 17 and 19mm. In contrast, BioRad discs provide a lower overall rate of error with both ME and VME errors. All strains displaying an inhibition diameter between 17 and 23mm should be tested by a second method.

In conclusion, DDM lack of accuracy for strains displaying borderline inhibition zone diameter regarding the breakpoint's guidelines. We suggest applying the concept of the area of technical uncertainty for standard DDM. The strains displaying a diameter within the ATU should be tested by another method. The significance of colonies within the inhibition zone diameter should be explored.

Abbreviations

eXDR: extremely drug resistant micro-organisms; CPE: carbapenemase producing *Enterobacterales*; DDM: disc diffusion method; ATU: area of technical uncertainty; ESBL-E: extensive spectrum beta-lactamase producing-*Enterobacterales*; 3GC-S: third generation cephalosporin susceptible-strain; 3GC-R: third generation cephalosporin resistant-strain; MIC: minimal inhibitory concentration; COPE: cephalosporinase overproducing *Enterobacterales*; ME: major errors; VME: very major errors.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

AH and EF conceived the study. AH coordinated recruitment, acquisition of study data and perform the statistical analyses. AH and EF contributed to the analysis of study data. All authors contributed to the interpretation of the data and approved the final version of the manuscript after critical review.

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Tables

Table 1. Number of isolates included for each species

Bacterial species	Number of isolates	% of isolates
Escherichia coli	459	51,69%
Klebsiella pneumoniae	134	15,09%
Enterobacter cloacae complex	126	14,19%
Klebsiella aerogenes	38	4,28%
Citrobacter freundii complex	29	3,27%
Klebsiella oxytoca	18	2,03%
Morganella morganii	17	1,91%
Hafnia alvei	16	1,80%
Proteus mirabilis	16	1,80%
Serratia marcescens	12	1,35%
Citrobacter koseri	8	0,90%
Klebsiella variicola	5	0,56%
Proteus vulgaris	5	0,56%
Salmonella spp.	3	0,34%
Providencia stuartii	1	0,11%
Raoultela ornithinolytica	1	0,11%

Table 2. MIC distribution and categorization error rate by DDM

	Overall isolates				Inhibition zone diameter 17-23mm			
	No. of isolates	Overall error	Of which ME*	Of which VME**	No. of isolates	Overall error	Of which ME*	Of which VME**
Overall	888	94 (10,6%)	68 (72,3%)	26 (27,7%)	488	75 (15,4%)	53 (70,7%)	22 (29,3%)
3GC-S	240	**17 (5,2%)**	13 (76,5%)	4 (23,5%)	146	13 (9,1%)	10 (76,9%)	3 (23,1%)
3GC-R	548	**77 (14,1%)**	55 (71,4%)	22 (28,6%)	333	62 (18,6%)	43 (69,4%)	19 (30,6%)
BioRad	657	57 (8,7%)	34 (59,6%)	23 (40,4%)	337	49 (14,5%)	28 (57,1%)	21 (42,9%)
I2a	231	37 (16,0%)	**34 (91,9%)**	3 (8,1%)	151	26 (17,2%)	**25 (96,2%)**	1 (3,8%)

* ME: major error; VME: very major error.

Figures

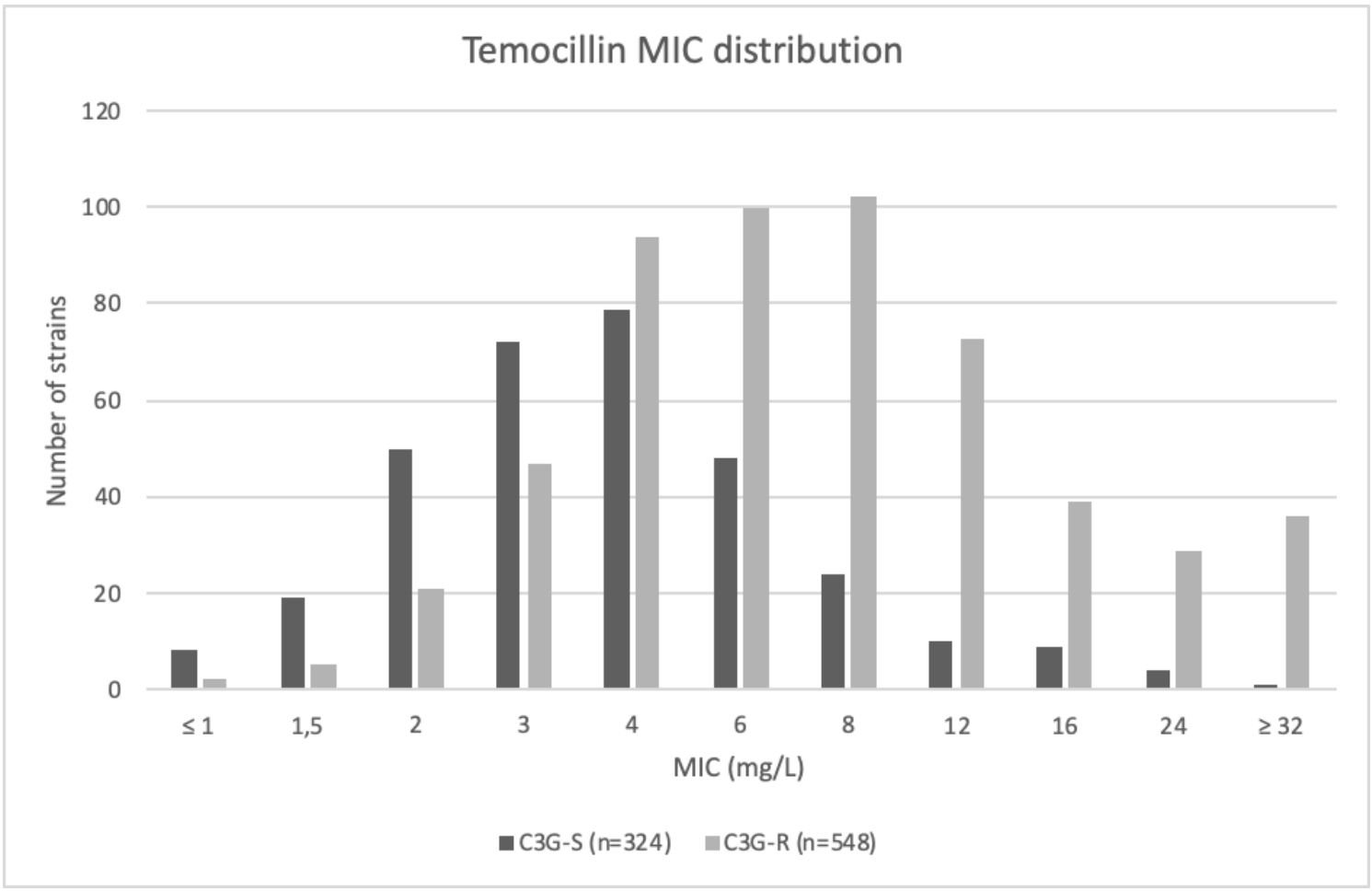

Figure 1

Temocillin MIC distribution assessed by E-TEST

Inhibition zone Diameter (mm)

Etest MIC determination (mg/L)	≤10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	≥30	*Total*
≥64	12																					*12*
48			1								1											*5*
32	3	3	6	1	3	1				1												*20*
24	5		5	2	8	6	5	1	2	2	1											*34*
16	2	1	2		3	3	16	9	5	4	3				1							*49*
12	2			1	2	8	7	13	20	12	10	5	1	1			2				1	*85*
8	2			2	1	1	3	7	9	14	37	20	15	8	5	3	1	1				*128*
6	1				2		2	2	1	8	31	26	27	16	17	10	2	4	1	1	1	*152*
4	1								3	6	21	15	27	27	23	15	24	6	2	2	2	*175*
3	2							1		1	5	9	16	16	18	19	20	8	4	5	5	*122*
2											3	1	6	10	5	11	13	9	5	4	3	*72*
1,5									1		1		3	2	3	1	3	3	3	2		*24*
≤1														2	1	3	1				2	*10*
Total	*30*	*4*	*14*	*6*	*19*	*19*	*33*	*33*	*41*	*48*	*113*	*76*	*95*	*82*	*73*	*62*	*66*	*31*	*15*	*14*	*14*	*888*

Figure 3

Distribution Etest MIC and zone diameter