TEMOCILLIN SUSCEPTIBILITY TESTING WITH 6 DIFFERENT METHODS ON *ENTEROBACTER AEROGENES*

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ABSTRACT

Background: Temocillin is a ticarcillin derivate with increased β-lactamase stability, active against Enterobacteriaceae. We compared the performance of 5 different methods for temocillin susceptibility testing on *E. aerogenes* strains with the broth microdilution (BM) method.

Methods: On 151 consecutive isolates of *E. aerogenes* collected in 5 Belgian Hospitals, temocillin susceptibility testing with 6 different methods was performed: BM as reference method, disk diffusion with paper disks (Oxoid)(DP), disk diffusion with Rosco tabs (Neo-Sensitabs ®)(DR), E-test ® strips (AB Biodisk)(ET), Phoenix (BD)(PH) and Vitek 2 (bioMérieux)(VI). Results were categorized as susceptible (S), intermediate (I) or resistant (R). Discrepancies in SIR-category were categorized as minor (1 step difference), major (BM S, experimental method R) and very major error (BM R, experimental method S). Association was further analyzed by means of odds ratios. Agreement was analyzed by the κ-statistic. The Pearson correlation was calculated between the experimental methods and BM for MIC-results.

Results: Minor, major and very major errors were seen in respectively 11, 6 and 1% of results with DP, in 5, 0 and 5% with DR, in 0, 7 and 4% with ET, in 0, 49 and 0% with PH and in 0, 7 and 5% with VI. All methods were significantly associated with BM, however odds ratios were only slightly above 1, indicating a weak association. Agreement was found to be rather low. The 3 methods with MIC results were significantly correlated by Pearson correlation; but the correlations were not very high. E-test was the strongest correlated with BM.

Conclusions: Major discrepancies with BM were seen for all experimental methods. Agreement and association were fairly low. For MIC determination E-test looks the most reliable of the tested methods.

INTRODUCTION

Temocillin is a semisynthetic 6-a-methoxy derivate of ticarcillin active on Enterobacteriaceae and stable against β-lactamases, including AmpC and some extended spectrum β-lactamases (ESBL). Despite this interesting feature, the lack of in vitro and in vivo studies hampers the breakthrough of this antibiotic in clinical use. Temocillin is available in Belgium as Negaban ® (licensed to Eumedica). It is being re-launched in the UK [1] and was approved recently in the USA for treatment of B. cepacia lung infection in cystic fibrosis [2].

During the year of 2005, several new commercial systems (ET and new panels on PH and VI) became available for routine susceptibility testing for temocillin. Before setting up more and larger in vivo studies, it seemed mandatory that first these new potentially promising techniques were validated in relation to the reference method (BM).

In this study we compared the performance of 5 different methods for temocillin susceptibility testing on *E. aerogenes* with the broth microdilution (BM) method on 151 consecutive E. aerogenes strains, collected in the first 3 months of 2005 in 5 Belgian Hospitals.

METHODS (1)

Bacterial strains: origin and identification.
In the first 3 months of 2005, 160 consecutive non duplicate isolates of *E. aerogenes* were collected in 5 Belgian hospitals (Onze-Lieve Vrouw Hospital, Aalst, Imelda Hospital, Bonheiden, Hospital Oost-Limburg, Genk, Sint-Lucas Hospital, Ghent, Virga Jesse Hospital, Hasselt) from clinical samples of hospitalized and ambulant patients.
After primary bacterial identification by routine procedures of each laboratory, strains were frozen at ≤ –70°C till further analysis.
Identification of the strains was confirmed by Phoenix (Becton-Dickinson, Sparks, MD, USA) using the Combo panel (NMIC/ID-51), and by Vitek 2 (bioMérieux, Marcy l'étoile, France) using the Vitek 2 colorimetric GN Card. In case of any discrepancy, strains were identified by API 20E (bioMérieux, Marcy L'Etoile, France) and by sequencing of a 570 base pair long amplicon of the gene coding for the small subunit of 16S ribosomal RNA and a blast search of the obtained sequences at the site of the National Centre for Biotechnology Information (http://www.ncbi.nih.gov/BLAST).Nine strains were excluded because of false identification or contamination.

Antibiotic susceptibility testing.
Broth microdilution method
All strains were tested using the CLSI reference broth microdilution method [3]. Inocula prepared in Mueller Hinton broth (Becton Dickinson, Sparks, MD, USA) to achieve 5 × 105 CFU/ml as final concentration, were incubated 18 to 24 h at 37°C with a range of 1 to 512 mg/L of temocillin.
E-test
The minimal inhibitory concentrations (MICs) for temocillin were measured on Mueller-Hinton agar (Oxoid, Wesel, Germany) with E-test (AB Biodisk, Solna, Sweden) according to the manufacturers' instructions.
Disk diffusion
Susceptibility testing by disk diffusion was performed according CLSI performance standards with 30 µg temocillin paper disks (Oxoid, Basingstoke, UK) on Mueller Hinton II agar (bioMérieux, Marcy-l'Etoile, France) and 30 µg temocillin Neo-Sensitabs ® (Rosco Diagnostica A/S, Taastrup, Denmark) on Mueller Hinton (Oxoid, Wesel, Germany). After 16-18 hours of incubation in ambient air at 35°C, plates were read visually and by Osiris camera (Bio-Rad, France).
Phoenix
Susceptibility testing on the Phoenix system was performed with the Phoenix Combo panel (NMIC/ID-51) (Becton-Dickinson, Sparks, MD, USA) according to the manufacturers' instructions.
Vitek 2
On the Vitek 2 system, the AST-N045 card (bioMérieux, Marcy-l'Etoile, France) was used for susceptibility testing according to the recommendations of the manufacturer.
Interpretation of the results
MIC results were categorized according to the breakpoints published by Fuchs and colleagues: susceptible: < 16 mg/L, resistant: > 32 mg/L. Results of temocillin paper disks were interpreted according to the breakpoints published by Fuchs in the same article: susceptible > 19 mm; intermediate: 16 – 18 mm; resistant: < 15 mm [4]. Results of temocillin Neo Sensitabs ® were interpreted according to the breakpoints provided by the manufacturer: susceptible: > 18 mm; intermediate: 15 – 17 mm; resistant: < 14 mm [5].
For the Phoenix and Vitek 2 systems, the "raw" MIC values were used, not taking in account any suggested adapted interpretations by the expert systems.

METHODS (2)

Quality control
Quality control strains (*Escherichia coli* ATCC 25922 and 35218) were included in each run for each method.
For E-test the quality control acceptation limits of the package insert supplement were used: 4 – 16 mg/L for *E. coli* ATCC 25922 and 2 – 8 mg/L for *E. coli* ATCC 35218.
For the interpretation of the quality control results of temocillin disk diffusion, we used the recommendations of the manufacturers: for temocillin paper disks, 20 – 24 mm (*E. coli* ATCC 25922) and 24 – 26 mm (*E. coli* ATCC 35218) [6]; for temocillin Neosensitabs™, 18 – 24 mm (*E. coli* ATCC 25922) and 20 – 26 mm (*E. coli* ATCC 35218) [7].
Statistics
Statistical analysis was supervised by the Center for Statistics, University of Hasselt, Agoralaan 1, D-building, B-3590 Diepenbeek, Belgium, annouschka.laenen@uhasselt.be.

In our study the checklist with STARD criteria was used wherever applicable [8].

RESULTS

In the study 160 observations were made, of which 9 were problematic and therefore deleted, so that the analysis is based on 151 observations. The scheme in **Table 1** was used to categorize the measurements [9]. For the Phoenix technique, a result '>16' was considered as Resistant.
Table 2 summarizes the frequency distribution of the six measurements over the three susceptibility categories:

Table 1

	Resistant	Intermediate	Susceptible
Rosco discs	≤ 14	15 - 17	≥ 18
paper discs	≤ 15	16 - 18	≥ 19
Phoenix	≥ 32		≤ 16
Vitek			
E-test			
microdilution			

Table 2

	Resistant	Intermediate	Susceptible
Rosco discs	1	8	142
paper discs	14	16	121
Phoenix	85	0	66
Vitek	13	0	138
E-test	16	0	135
microdilution	11	0	140

A difference of one step between the experimental method and the reference method (all combinations S-I and R-I) is considered as a 'minor discrepancy'. When the reference method says S and the experimental method says R, we call it a 'major discrepancy'. Whenever the reference method gives R and the experimental method gives S, it is called a 'very major discrepancy'. According to this terminology the discrepancies between the five experimental methods and the reference method are given in **Table 3**.

Table 3

	Minor	Major	Very major
Rosco discs	8 (5 %)	0 (0 %)	8 (5 %)
paper discs	16 (11 %)	9 (6 %)	2 (1 %)
Phoenix	0 (0 %)	74 (49 %)	0 (0 %)
Vitek	0 (0 %)	10 (7 %)	8 (5 %)
E-test	0 (0 %)	11 (7 %)	6 (4%)

DISCUSSION (1)

Agreement [10,11]

Table 4 gives the weighted kappa-statistic indicating the agreement between the respective technique and the reference method based on microdilution. Besides the kappa-statistic, also the ASE (an indicator of variance) and the 95% confidence interval (CI) for the kappa-statistic are given.
Table 5 gives a guideline to interpret the value of a kappa-statistic [12]. According to these guidelines, the agreement is poor between the techniques Phoenix and Vitek on one hand and the microdilution method on the other hand. All other techniques have a 'fair' agreement with the reference method, even though for the Rosco discs this can be considered as borderline. The paper discs have the highest agreement with the reference method and also the confidence interval is better (narrower) then the one for the E-test.

Table 4

	Kappa	ASE	95% CI
Rosco discs	0.214	0.120	-0.021; 0.449
paper discs	0.362	0.096	0.174; 0.551
Phoenix	0.115	0.034	0.048; 0.182
Vitek	0.186	0.123	-0.055; 0.427
E-test	0.311	0.125	0.066; 0.556

Table 5

Kappa	Strength of agreement
< 0.2	Poor
> 0.2 ≤ 0.4	Fair
> 0.4 ≤ 0.6	Moderate
> 0.6 ≤ 0.8	Good
> 0.8 ≤ 1	Very good

Association

Correlation

The experimental methods Phoenix, Vitek and E-test, and the reference method are strictly speaking not measured continuously, but the outcomes consist of several (minimum 4) ordered categories that can be converted according to **Table 6**. In practice, ordered categories with four or more levels are regularly analyzed as continuous variables.

Table 6

Phoenix	Vitek	E-test	Reference	converted to...
2			2	2
4	<=4	4	4	4
8	8	8	8	8
16	16	16	16	16
>16	>=32	32	32	32
		48	64	48
		64		64
		96		96

DISCUSSION (2)

We use the converted values to calculate the Pearson-product moment correlation between the experimental methods and the reference method. **Table 7** gives the results. (The p-value indicates the probability that, purely by chance, two independent/uncorrelated variables will take the precise combination of values that was observed. Whenever this chance is smaller than 5% or 0.05, we consider the correlation as significant.) The three methods are significantly correlated with the reference method at the 5% level. E-test has the strongest correlation with the reference method. However correlations are generally rather low.

Table 7

Experimental method	Pearson correlation	p- value
Phoenix	0.394	0.001
Vitek	0.240	0.0305
E-test	0.502	<0.0001

Association between categorical variables

We fitted the proportional odds model separately for each of the techniques. The categorical outcomes (R, I, S) of the experimental techniques are taken as the response variable and the raw (uncategorized) measurement outcomes of the microdilution method are taken as the explanatory variable. The distribution of the raw measurements for the microdilution method are as in **Table 8**.

Table 8

observed value	Frequency
2	10
4	24
8	61
16	45
32	10
64	1

Table 9

	Odds ratio	95% CI
Rosco discs	1.084	1.026; 1.045
paper discs	1.182	1.114; 1.255
Phoenix	1.201	1.112; 1.296
Vitek	1.121	1.053; 1.194
E-test	1.146	1.076; 1.221

We summarize the results for the five models by giving the odds ratios and the confidence intervals for the odds ratios (**Table 9**). An odds ratio of 1 indicates that the two variables are independent. The further away the value is from 1, in either direction, the stronger is the association between the two variables. We conclude that two variables are independent (not related) when the confidence interval contains the value 1.

All odds ratios are slightly above 1 and none of the confidence intervals contains 1. This means that we can conclude that all of the experimental techniques are significantly associated with the reference technique. In contrast to the results on agreement, the results of the Phoenix technique are the strongest related to the reference method. However the association for the paper discs method is not much lower and the confidence interval is better (narrower). In general the odds ratios are all rather similar, and generally low (not much above 1).

CONCLUSIONS

- **Major discrepancies with BM were seen for all experimental methods.**
- **Agreement and association were fairly low.**
- **For MIC determination E-test looks the most reliable of the tested methods.**

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