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Population Analysis of *Escherichia coli* Isolates with Discordant Resistance Levels by Piperacillin-Tazobactam Broth Microdilution and Agar Dilution Testing

Carole Shubert,[a] Jen Slaughter,[a] David Creely,[a] Alex van Belkum,[b,c] Jean Pierre Gayral,[b] William Michael Dunne,[d] Gilles Zambardi,[b] Dee Shortridge[a]

bioMérieux, Inc., R&D Microbiology, St. Louis, Missouri, USA[a]; bioMérieux SA, R&D Microbiology, La Balme-les-Grottes, France[b]; Erasmus Medical Center, Medical Microbiology and Infectious Diseases, Rotterdam, The Netherlands[c]; bioMérieux, Inc., R&D Microbiology, Durham, North Carolina, USA[d]

Population analysis was performed for 42 *Escherichia coli* isolates to determine whether heterogeneity of resistance was a factor in piperacillin-tazobactam category differences between agar dilution and broth microdilution. Of 20 isolates discordant between methods, 80% were heterogeneous. Of 22 isolates in agreement, 59% were homogeneous. Heterogeneity and homogeneity rates for those in agreement were significantly different from those that were discordant (*P* value, 0.010). Heterogeneity of resistance expression appears to be an important factor in category differences observed between broth microdilution and agar dilution for piperacillin-tazobactam.

Interpretation of antimicrobial susceptibility tests can be subjective, even for established reference methods. Various *in vitro* methods to assess susceptibility have been developed, and conditions can vary widely during measurements. This diversity can cause differences in the metabolic status of bacterial cells and thus their reactivity to antibiotics, implying that results from different methods can vary per strain, as was recently seen in a multicenter study of *Pseudomonas aeruginosa* (1). Methodological differences can lead to very major errors (VMEs) (reference method resistant and comparator susceptible), which may lead to treatment failure. Major errors (MEs) (reference method susceptible and comparator resistant) can also occur and, although less serious therapeutically, can lead to disqualification of otherwise useful antimicrobials. The frequency of VMEs and MEs depends on the methods used and organism/antimicrobial combinations. However, certain drugs (especially combinations) are often problematic. Root causes for such differences are usually unknown, but clear differences between piperacillin-tazobactam (TZP) broth microdilution (BMD) and agar dilution (AD) results were observed for isolates of several species (2–5). Although differences between BMD and AD have been observed for other species, the population analysis discussed here was limited to *Escherichia coli*. We recently demonstrated that this aberrant phenotype can be clonal in *E. coli* (5). In this study, isolates were tested by population analysis (PA) in order to determine whether heterogeneity of resistance expression was a factor in the category differences observed between BMD and AD for TZP.

A subset of 42 *E. coli* isolates was selected from a larger set of *Enterobacteriaceae* obtained for the purpose of studying TZP susceptibility (5). The isolates were obtained globally from as many geographically diverse locations as possible. Isolates in agreement as well as those in disagreement between BMD and AD were included. Strain characteristics are included in Table 1.

BMD and AD TZP susceptibility testing was performed according to CLSI guidelines (6) and was done in triplicate. MICs were determined by examining the growth for each of the three replicates for each isolate and method. The lowest concentration that completely inhibited visible growth as detected by the unaided eye was recorded as the MIC. If two of three results were the same, that MIC was used as the composite ("voted") result. If three results differed, the middle result was used. CLSI breakpoints (7) were applied to determine category interpretations (susceptible, intermediate, or resistant) (6). BMD and AD category interpretations were determined and compared for each isolate.

PA was performed by testing each isolate on a series of agar dilution plates, prepared without drug (growth control), and with piperacillin in serial doubling dilutions between 1 and 256 μg/ml plus tazobactam at a fixed concentration of 4 μg/ml (8, 9). Plates were inoculated with 100 μl of a 0.5 McFarland suspension (in 0.45% saline), and the inoculum was spread using a disposable bent inoculator and a manual plate spinner. Plates were incubated for 16 to 20 h at 35°C. The number of colonies on each plate in the series per isolate was recorded. Results were interpreted as follows: if MICs of the population spanned more than three concentrations, the population was considered to be heterogeneous; if MICs spanned no more than three concentrations, the population was considered to be homogeneous.

Chi-square analysis was used to determine statistical significance. A *P* value of ≤0.05 was considered significant.

Molecular testing was performed using crude lysates, and PCR was performed to determine the presence of bla_{TEM}, bla_{SHV}, the bla_{OXA-1} group, bla_{CTX-M}, and plasmid-mediated bla_{AMP-C}. PCR products for bla_{TEM} were sequenced. The PCR assay for plasmid-mediated bla_{AMP-C} was able to detect six different families of plasmid *ampC* forms (5, 10).

DiversiLab (bioMérieux, Marcy l'Etoile, France) strain typing

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Address correspondence to Carole Shubert, carole.shubert@biomerieux.com.

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TABLE 1 Results for all isolates[a]

Tally	Stock no.	Isolate origin	β-Lactamase(s)	Composite BMD (μg/ml)	Composite AD (μg/ml)	CAT between BMD and AD?	Population analysis interpretation
1	904944	Canada	bla_{TEM-1}	128	8	N	HET
2	907357	Austria	bla_{TEM-1}	128	8	N	HET
3	907442	Scotland	bla_{TEM-1}	128	16	N	HET
4	907672	United States	bla_{TEM-1}	>256	64	N	HET
5	907700	United States	bla_{TEM-1}	>256	8	N	HET
6	907710	United States	bla_{TEM-1}, bla_{CTX-M}	>256	8	N	HET
7	907892	United States	bla_{TEM-1}	>256	8	N	HET
8	908519	United States	bla_{TEM-1}	128	64	N	HET
9	908521	United States	bla_{TEM-1}	128	32	N	HET
10	908522	United States	bla_{TEM-1}	256	64	N	HET
11	908555	Italy	bla_{TEM-1}	64	16	N	HET
12	908585	Spain	bla_{TEM-1}	>256	64	N	HET
13	908675	U.K.	bla_{TEM-1}	256	64	N	HET
14	908691	South Korea	bla_{TEM-1}, bla_{CTX-M}	64	8	N	HET
15	907355	Austria	bla_{TEM-1}	256	16	N	HET
16	907402	Scotland	bla_{TEM-1}	256	8	N	HET
17	907035	United States	bla_{TEM-1},bla_{CIT}	128	64	N	HOM
18	907715	United States	bla_{TEM-1}	256	32	N	HOM
19	907889	United States	bla_{TEM-1}	64	1	N	HOM
20	908632	Thailand	bla_{CTX-M}	32	16	N	HOM
21	905162	Canada	bla_{TEM-1}	2	4	Y	HET
22	907163	Germany	bla_{TEM-1}	>256	>256	Y	HET
23	907446	Scotland	bla_{TEM-1}	4	2	Y	HET
24	907701	United States	bla_{TEM-1}	>256	>256	Y	HET
25	907713	United States	bla_{TEM-1}	2	2	Y	HET
26	908541	Germany	bla_{TEM-1}	>256	>256	Y	HET
27	908573	China	bla_{TEM-1}, bla_{CTX-M}, bla_{SHV}	>256	>256	Y	HET
28	908616	Hong Kong	bla_{TEM-1}	>256	256	Y	HET
29	908624	South Africa	bla_{TEM-1}	256	128	Y	HET
30	904942	Canada	bla_{TEM-1}	>256	>256	Y	HOM
31	907075	United States	bla_{TEM-40}	>256	>256	Y	HOM
32	907149	Germany	bla_{TEM-1}	>256	>256	Y	HOM
33	907268	Germany	bla_{SHV}	>256	>256	Y	HOM
34	907374	Austria	bla_{OXA-1}	2	4	Y	HOM
35	907384	Scotland	bla_{TEM-1}, bla_{CIT}	128	128	Y	HOM
36	907578	United States	bla_{TEM-1}, bla_{FOX}	>256	>256	Y	HOM
37	907606	United States	bla_{TEM-1}, bla_{CIT}	>256	256	Y	HOM
38	907608	United States	bla_{CTX-M}	>256	>256	Y	HOM
39	907720	United States	bla_{SHV}	>256	>256	Y	HOM
40	908524	United States	bla_{TEM-1}	>256	>256	Y	HOM
41	908525	United States	bla_{TEM-1}, bla_{OXA-1}, bla_{CTX-M}	>256	>256	Y	HOM
42	103934	QC ATCC 35218	bla_{TEM-1}	1	2	Y	HOM

[a] CAT, categorical agreement (i.e., susceptible, intermediate, or resistant); Y, yes; N, no; HET, heterogeneous; HOM, homogeneous; QC, quality control strain.

was performed on all isolates according to the manufacturer's instructions. DiversiLab uses repetitive sequence-based PCR, which allows for the amplification of many differently sized fragments (amplicons) representing the DNA within noncoding, repetitive sequences in the genome. The arrangement of these fragments shows specific genotypic differences and therefore can be used to discriminate bacteria at the strain level.

A summary of results is shown in Table 1.

Of 42 total isolates, 22 were in categorical agreement between BMD and AD and 20 were not. Of the 22 in agreement, 13 (59.1%) were homogeneous and 9 (40.1%) were heterogeneous. Of the 20 not in agreement, 16 (80%) were heterogeneous and 4 (20%) were homogeneous. The rates of heterogeneity for those in agreement compared to those in disagreement were significantly different ($P = 0.010$). The rates of homogeneity were also significantly different ($P = 0.010$) between those in agreement and those in disagreement. Higher MICs were observed for BMD when BMD and AD were discordant. Excluding one-dilution differences between BMD and AD (which were within essential agreement), there were 18 isolates with TZP MICs more resistant by BMD and no isolates more resistant by AD. It cannot be determined with certainty whether BMD or AD correlates better with clinical outcome; these data have not been recorded. The conservative approach

was to consider BMD less risky regarding patient safety since BMD MICs are higher when the two methods are discordant.

The majority of *E. coli* isolates studied (28/42; 66.7%) harbored only the bla_{TEM-1} gene; 22 of 24 heterogeneous isolates harbored only bla_{TEM-1}. The homogeneous isolates contained a wider variety of β-lactamases, some in combination with bla_{TEM-1}, bla_{SHV} alone, or the bla_{OXA-1} group alone. The remaining two heterogeneous isolates harbored bla_{TEM-1} and bla_{CTX-M}. Sequencing was done only on bla_{TEM} isolates. Since an *E. coli* isolate with a basal level of bla_{TEM-1} is not expected to be TZP resistant, further investigation of the resistance mechanism for these isolates was initiated. Possible explanations for the observed resistant phenotype include hyperproduction of β-lactamase, higher overall β-lactamase level due to the number of β-lactamases present per isolate, variation in periplasmic concentration of β-lactamase, efflux alterations, and/or porin mutations (11–15).

DiversiLab strain typing showed that although there were similarities between some strains, there was no unique clone responsible for either heterogeneity of resistance or discordant results (data not shown).

Data were also analyzed with the application of EUCAST breakpoints (data not shown). TZP EUCAST breakpoints are lower than CLSI breakpoints, and more isolates were in category agreement between BMD and AD with EUCAST breakpoints than with CLSI breakpoints. For those in category agreement with EUCAST breakpoints, there was an even split between heterogeneous and homogeneous isolates (15 of each). However, for those that were discordant between BMD and AD, a higher percentage were heterogeneous (10/12, or 83.3%) than homogeneous (2/12, or 16.7%). Therefore, the trend toward a higher percentage of heterogeneity for isolates discordant between BMD and AD was still present with EUCAST breakpoints.

The data presented support that TZP resistance differences between BMD and AD are at least in part due to heterogeneity of resistance expression. The exact mechanism of differences observed requires further investigation, although delayed growth of the resistant subpopulation may be responsible.

It was recently shown that a factor in the outcome of bacteremia episodes was the TZP MIC of the causative strains of *E. coli* (16), thus underscoring the importance of accurate MICs. We showed that population heterogeneity is correlated to TZP susceptibility variability between reference methods, thereby demonstrating that susceptibility testing can be confounded by basic biological characteristics of the microbial cells. It is important to be aware that for some specific strains, even highly valued susceptibility reference methods may generate conflicting results.

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