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Effect of Zinc Concentration in Mueller-Hinton Agar on Susceptibility of *Pseudomonas aeruginosa* to Imipenem

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The susceptibility of *Pseudomonas aeruginosa* to imipenem has been shown to vary according to zinc concentration in the media. MICs of imipenem for 68 unique clinical isolates of *P. aeruginosa* were determined in media supplemented with zinc at concentrations between 0.5 and 6.0 μg/ml. In agar containing up to 3 μg of zinc/ml, 75 to 82% of the strains were susceptible to imipenem at an MIC of ≤4 μg/ml. In agar supplemented to contain 6 μg of zinc/ml, however, only 40% of the strains were susceptible to imipenem. Manufacturers should ensure that the concentration of zinc in commercial media is below 3 μg/ml to avoid false classification of isolates as resistant to imipenem.

Imipenem (*N*-formimidoyl thienamycin), a member of the carbapenem class of antibiotics, has activity against a broad spectrum of organisms that includes *Pseudomonas aeruginosa*. Traditionally, serious infections due to this organism require the administration of two effective antimicrobial agents to achieve cure and to help prevent the development of resistance during treatment (4). Accurate detection of resistance of *P. aeruginosa* to imipenem by the clinical microbiology laboratory is critical for proper use of this antibiotic (9).

Unfortunately, in vitro testing of imipenem has been problematic, partly because of the instability of this compound in the commercially prepared dry-format, predried broth, and frozen microdilution panels that are used by many laboratories for susceptibility testing (1, 3, 5, 8). Baron and Hindler showed that the bioactivity of imipenem in these panels is dependent upon the type of medium used and on the time and temperature of incubation (1). Other investigators found additional factors that affect the reliability of imipenem bioactivity tests: variations in lots of Mueller-Hinton broth (MHB), multiple passaging of the control strain of *P. aeruginosa*, and moisture in stored dry-format panels (5, 8, 9).

A study by Cooper and colleagues reported that zinc concentration in commercial media influenced the susceptibility of *P. aeruginosa* and other gram-negative bacilli to imipenem (2). They found that a Mueller-Hinton agar (MHA) with a higher zinc concentration (2.62 μg/ml) was associated with a higher MIC of imipenem for *P. aeruginosa*, reaching concentrations that are greater than or equal to the susceptibility breakpoint of 16 μg/ml established by the National Committee for Clinical Laboratory Standards (NCCLS), resulting in misclassification of susceptible strains as resistant (2). However, it appears that the changes in MICs were within a onefold dilution for many strains and within the inherent error of the assay. In addition, Cooper et al. tested media with differences other than zinc concentration alone obtained from different manufacturers. In this study, we attempted to document the findings of Cooper et al. and to determine whether higher zinc concentrations in MHA independently increase the MIC of imipenem for *P. aeruginosa*.

(This data was presented in part at the 96th General Meeting of the American Society for Microbiology held in New Orleans, La., 19 to 23 May 1996.)

Methods and materials. Sixty-eight unique clinical strains of *P. aeruginosa* were collected from The Medical Center of Central Massachusetts, Worcester, Mass. Thirty-four strains were collected from 3 October 1986 to 25 June 1993 and were taken from our frozen collection, and 34 strains were recent clinical isolates collected from 19 November 1994 to 8 February 1995. Control strains, *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922, were tested throughout the study. Bacterial isolates were stored at −70°C in semisolid brain heart infusion agar (Difco Laboratories, Detroit, Mich.). Isolates were grown overnight at 35°C on Trypticase soy agar with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.).

Plates for agar dilution testing were prepared by using imipenem reference powder (Merck and Co., Inc., West Point, Pa.; lot 7058A) and MHB plus 17 g of Bacto-Agar (Difco Laboratories, Detroit, Mich.) per liter. Concentrations of imipenem tested were 0.06 to 125 μg/ml. The media used in the study were BBL MHB (BBL Microbiology Systems) and Difco MHB (Difco Laboratories). The Difco brand of MHB contained the lowest concentration of zinc and was supplemented with zinc acetate dihydrate (Sigma Chemical Company, St. Louis, Mo.) to achieve final concentrations of 6.0, 3.0, 2.0, 1.0, and 0.5 μg of zinc/ml in the agar. An aliquot of each concentration was sent to Merck and Co., where the actual zinc content in the broth was determined by an inductively coupled plasma-atomic emission spectroscopy method. Solutions containing imipenem were used immediately to avoid deterioration before testing.

Agar dilution testing was performed in accordance with NCCLS guidelines (7). Colonies of each bacterial isolate were suspended in MHB to match a 0.5 McFarland standard. The suspensions were then diluted 1:10 and inoculated onto antibiotic-containing agar plates by using a Steers replicator to deliver approximately 10^4 CFU per spot. The plates were incubated at 35°C for 16 to 20 h. MICs were determined as the lowest concentration of antimicrobial agent that completely inhibited growth, disregarding a single colony or a faint haze caused by inoculum (7). Agar dilution testing of imipenem against 68 clinical isolates of *P. aeruginosa* was conducted si-

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TABLE 1. Susceptibility of 68 strains of *P. aeruginosa* to increasing concentrations of imipenem when tested in media containing measured amounts of zinc

Imipenem concn (μg/ml)	% of strains susceptible[a] with zinc concn (μg/ml) of:					
	0.2	0.5	1	2	3	6
0.25	2	2	0	0	0	0
0.5	2	2	0	2	0	0
1.0	47	44	43	25	7	0
2	75	78	71	57	51	3
4	82	81	82	77	75	40
8	85	87	85	87	87	91
16	96	96	96	96	97	100
32	100	100	100	100	100	100

[a] Cumulative percentage of 68 strains.

multaneously on BBL MHA, Difco MHA, and Difco MHA supplemented with 6.0, 3.0, 2.0, 1.0, and 0.5 μg of zinc/ml.

Results. The actual zinc concentration was measured by inductively coupled plasma-atomic emission spectroscopy. The zinc content of Difco MHA was 0.2 μg/ml. In zinc-supplemented media, the measured zinc content was ±18% of the target concentration for all solutions tested.

Table 1 shows the cumulative percentages of 68 strains of *P. aeruginosa* that were susceptible to increasing concentrations of imipenem when tested with BBL MHA, Difco MHA unsupplemented with zinc, and Difco MHA supplemented with 0.5, 1.0, 2, 3, and 6 μg of zinc/ml. The percentage of isolates susceptible to imipenem at various concentrations was similar for Difco unsupplemented MHA (zinc concentration = 0.2 μg/ml) and for MHA supplemented with 0.5 μg of zinc/ml. There was no notable difference in susceptibility to imipenem when agar dilution testing was performed on media supplemented with zinc at concentrations of 0.5 versus 1.0 and 2 versus 3 μg/ml.

Table 2 illustrates the influence of various zinc concentrations in MHA on susceptibility of the 68 *P. aeruginosa* isolates to imipenem. The number of isolates that were resistant (9 to 15%) remained generally constant throughout the study. The most notable difference in susceptibility occurred when MHA was supplemented with 6 μg of zinc/ml, at which concentration 51% of the strains were classified as intermediate; in contrast, 3 to 12% of the strains were intermediate when tested in all other types of media with lower zinc concentrations. For classification as susceptible, 75 to 82% of the strains were susceptible to imipenem in all media except MHA supplemented with 6 μg of zinc/ml, in which only 40% of strains were susceptible. Figure 1 illustrates the degree of change in MIC of imipenem when *P. aeruginosa* isolates were tested by using MHA with zinc at concentrations of 3 and 6 μg/ml.

TABLE 2. Susceptibility of *Pseudomonas* isolates at various zinc concentrations

Concn (μg/ml) of zinc in Difco medium	No. of strains (%)[a]		
	S	I	R
None (unsupplemented)	56 (82)	2 (3)	10 (15)
0.5	55 (81)	4 (6)	9 (13)
1.0	56 (82)	2 (3)	10 (15)
2.0	52 (76)	7 (10)	9 (13)
3.0	51 (75)	8 (12)	9 (13)
6.0	27 (40)	35 (51)	6 (9)

[a] S, susceptible; I, intermediate; R, resistant.

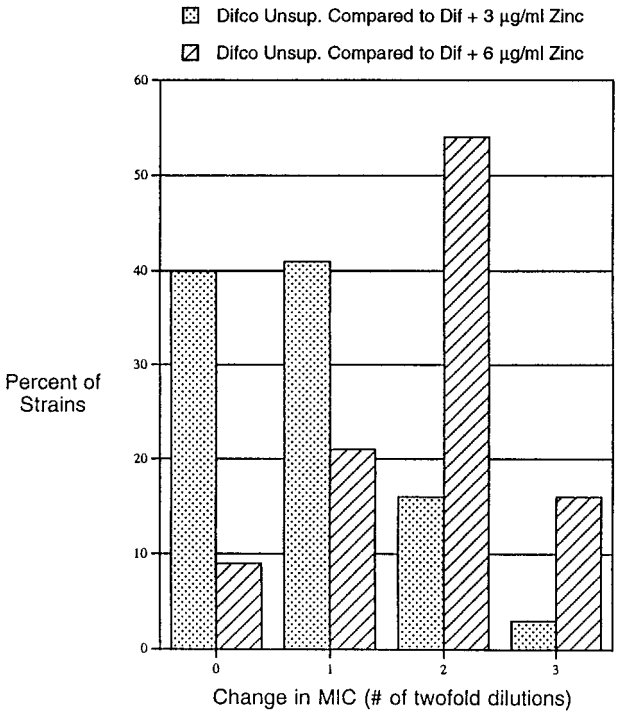

FIG. 1. Changes in MIC of imipenem for *P. aeruginosa* isolates in MHA with various zinc concentrations.

Discussion. Cooper and colleagues (2) reported that the zinc concentration in MHA can increase the MIC of imipenem for *P. aeruginosa*, often to concentrations that are greater than or equal to the susceptibility breakpoint of 16 μg/ml established by NCCLS (7). They noted that the effects of high zinc concentration varied in media from different manufacturers. Cooper et al. concluded that this finding was clinically significant despite the fact that the difference seen was a onefold dilution when the broth dilution method was used. Their conclusion was valid for strains for which the MIC was at the breakpoint of interpretive susceptibility criteria (between 4 and 16 μg/ml), where a shift of one twofold dilution can change the interpretation from "susceptible" to "intermediate" or from "intermediate" to "resistant." For 95% of the strains they tested, the MIC was less than or equal to 8 μg/ml when a low-zinc medium (Difco) was used, and for 93%, the MIC was less than or equal to 16 μg/ml when a medium with a higher zinc concentration (2.61 μg/ml; BBL) was used. Given the limits of reliability of agar dilution testing, we were concerned that the effect of zinc was unclear and that a concentration of 3 μg/ml did not produce a clinically significant difference in the MICs for most strains.

An increase in the amount of zinc in MHA correlates in vitro with an increase in the MIC of imipenem, but it is not known if zinc levels in humans influence imipenem in vivo antibacterial activity. The normal concentration of zinc in serum is 12 μmol/l or 0.8 μg/ml (6). The danger of misreporting strains as intermediate or resistant is that clinicians have limited antibiotic choices when treating patients with *Pseudomonas* infections and imipenem represents a necessary therapeutic option, often as a part of a multidrug regimen. However, at zinc concentrations of 3 μg/ml and below, differences in MICs were not clinically relevant, except for strains for which MICs are near the breakpoint for classification as intermediate or resistant.

Similarly, while at least 75% of all strains were inhibited by 4 μg of imipenem/ml in media supplemented with 0.5, 1, 2, and 3 μg of zinc/ml, fewer than half (40%) of the 68 strains were inhibited by 4 μg of imipenem/ml in MHA supplemented with 6 μg of zinc/ml. This suggests that by controlling the amount of zinc in media, manufacturers can prevent erroneous reporting by the microbiology laboratory of resistance to imipenem.

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