The Relationship Between Agar Thickness and Antimicrobial Susceptibility Testing

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Received: 27 September 2017 / Accepted: 17 October 2017 / Published online: 27 October 2017
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Abstract Antimicrobial susceptibility testing can be done using solid or liquid-based medium. Solid-based assays are easy and inexpensive; they are limited by not being as quantitative as liquid-based assays. Agar depth can influence the accuracy of plate-based assays and it is assumed the basis of this effect is antimicrobial agent diffusion. We tested this assumption by using ETEST® to quantitate the relationship between agar depth and minimum inhibitory concentration and zone of inhibition.

Keywords Antimicrobial susceptibility testing · Zone of inhibition · ETEST

Antimicrobial susceptibility testing (AST) is routinely performed in clinical laboratories to guide selection of appropriate antibiotic treatment for patients with infections, and in research laboratories studying antibiotic resistance. AST methods can be separated based upon use of liquid or solid media, and whether results are qualitative or quantitative [1].

Liquid medium assays use defined concentrations of antimicrobial agent to determine the minimum inhibitory concentration (MIC), which is the lowest concentration of the antimicrobial agent that inhibits growth in a defined condition [1]. Multiple automated systems are available for susceptibility testing [1], though they require specialized equipment. Although quantitative, the concentration range examined in liquid based assays is not continuous, resulting

in MIC accuracy being limited to the step differences in antimicrobial agent concentration between culture tubes.

The Kirby–Bauer disk diffusion method on 4 mm thick Mueller–Hinton agar is a common solid medium assay [2]. Diffusion from a disc containing a known amount of the antimicrobial agent added to a plate seeded with bacteria results in formation of a zone of inhibition (zoi). The edge of that zone occurs where the antimicrobial agent concentration is insufficient to prevent bacterial growth. Using CLSI guidelines [1, 3], the diameter of the zone allows a strain to be classified as sensitive, intermediate, or resistant to the drug. As described, this method provides categorical interpretative result only. Because solid medium assays are easy and low cost, they are commonly used.

Established procedures for manual AST assays improve reproducibility of results and accuracy of interpretations. Slight deviations from these practices can impact results. It is known that the zoi can be affected by the rate of bacteria growth and diffusion characteristics of the agent, but additional parameters, not recognized by many users of this technique, can affect results. These factors include the presence of multidrug resistance in tested strains [4], inconsistencies in inoculum preparation and application [5], disk positioning [6], and media and materials [7, 8]. The relevance of media and materials appears to be on agar thickness. Recommended solid medium used for diffusion testing is standard or cation-adjusted Mueller–Hinton agar at a depth of 4 mm [3, 9]. Davis and Stout [7] showed amount of agar poured, nonplanar plate bottoms, location of plate within a poured plate stack, and wedge-shaped agar all impact accuracy. Woolfrey et al. [8] identified Petri dish concavity as an accuracy variable. It is assumed that agar thickness and hydration impact zoi through influencing diffusion of the antimicrobial agent. To test this

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Table 1 Minimum inhibitory concentration (MIC) and zone of inhibition values (zone size) for ceftazidime (CAZ), meropenem (MEM) and minocycline (MH) at different depths of agar

Agar depth (mm)	Plate weight (g)	CAZ		Plate weight (g)	MEM		MH	
		MIC (µg/mL)	Zone size (mm)		MIC (µg/mL)	Zone size (mm)	MIC (µg/mL)	Zone size (mm)
4	31.13 (30.51, 31.07, 31.81)	0.75 (0.75, 0.75, 0.75)	34 (33, 34, 35)	32.44 (31.67, 31.77, 34.2)	6.67 (6, 6, 8)	19.33 (18, 19, 21)	5.33 (4, 4, 8)	19.33 (18, 19, 21)
6	42.06 (40.71, 42.28, 43.19)	1.17 (1.0, 1.0, 1.5)	30.67 (30, 31, 31)	42.193 (40.30, 43.00, 43.27)	14.67 (12, 16, 16)	16 (15, 16, 17)	10 (6, 12, 12)	16 (15, 15, 18)
8	51.71 (48.82, 51.73, 54.59)	1.33 (1.0, 1.33, 1.33)	29 (29, 29, 29)	51.91 (48.44, 49.82, 57.46)	21.33 (16, 24, 24)	14.67 (13, 15, 16)	8 (8, 8, 8)	18 (17, 18, 19)

Each value is the average of three replicates; the individual replicate values are given in parentheses

assumption requires having a quantitative gradient diffusion method. That method exists in the ETEST®.

ETESTs are plastic strips with antibiotic on one side and an MIC reading scale on the other; when placed onto solid media seeded with bacteria, the antimicrobial agent creates a continuous gradient of antibiotic [10]. The edge of bacterial growth next to the strip corresponds to the MIC. ETESTs combine the advantages of liquid and solid-medium based ASTs: ease of use, reduction in technical dilution errors, generation of MIC, and interpretive results. ETESTs are also expensive.

To examine the effects of variation of agar depth on AST determination, zoi's were correlated with agar thickness, as was done in previous studies, and then compared to MIC using ETESTs. Quantitative MIC results obtained from ETESTs allow more accurate assessment of the impact of agar thickness on zoi than reported in previous studies. AS149, a clinical strain of *Burkholderia multivorans* [11] was used to examine ceftazidime (CAZ), meropenem (MEM), and minocycline (MH). Mueller–Hinton plates of 1.7% agar (Fisher BioReagents Agar) were poured to a depth of 4, 6, and 8 mm. The gross weight of agar-containing plates was used to quantitate the amount of agar [the weight of empty plates varied by less than 0.3% (12.56 ± 0.033 g)]. Plate depth measurements were taken on the day of inoculation. Results are shown in Table 1.

As agar plate depth/weight increases, the MIC also increases and the zone of inhibition size decreases for ceftazidime and meropenem. For minocycline, the lowest MIC and largest zoi is observed in the thinnest plate, but the 6 mm plate has a higher MIC and lower zoi than does the 8 mm plate.

The effect of hydration on zone size and MIC was examined by using 4 mm and 8 mm plates for AST testing after being unstacked and closed on the bench top for 7 days. A comparison of MIC and zoi values (Table 2) with those given in Table 1 shows MIC inversely correlates, and zoi positively correlates, with plate weight.

The relationship between agar depth and MIC/zoi can be modeled through linear regression analysis. Figure 1 shows this data for MIC (panels a, c, e) and zone of inhibition (panels b, d, f) for each of the three antibiotics. In all cases, there is a positive slope between agar depth and MIC, and a negative slope between agar depth and zone of inhibition. The absolute slope varies with different antibiotics with meropenem having a higher slope than minocycline or ceftazidime.

One factor that likely influences the effect of agar depth on MIC and zoi is the agar diffusion coefficient of the antimicrobial agent. While not measuring the diffusion coefficient directly, we compared the molecular weight of the three antibiotics used in this study: meropenem has a lower molecular weight (383.46 g/mol) that minocycline

Table 2 Minimum inhibitory concentration (MIC) and zone of inhibition values (zone size) for meropenem (MEM) and minocycline (MH) on dehydrated agar plates

Agar depth (mm)	Plate weight (g)		MEM		MH	
	Prior to drying	On day of inoculation	MIC (μg/mL)	Zone Size (mm)	MIC (μg/mL)	Zone Size (mm)
4	32.53 (31.40, 32.93, 33.25)	30.052 (28.94, 30.42, 30.77)	6 (6, 6, 6)	21.33 (21, 21, 22)	4 (4, 4, 4)	21.33 (21, 21, 22)
8	52.629 (49.98, 50.87, 57.04)	50.056 (47.46, 48.29, 54.42)	18.67 (16, 16, 24)	15.33 (14, 16, 16)	8 (8, 8, 8)	17.33 (16, 18, 18)

Each value is the average of three replicates; the individual replicate values are given in parentheses. Plates were weighed after being poured (“prior to drying”) and then again after being left on the benchtop, unstacked and closed, at room temperature for 7 days (“On day of inoculation”). The “On day of inoculation” plates were used to generate the MIC and zone size results

Fig. 1 Linear regression of MIC values, given in μg/mL, for ETESTs and zone of inhibition values for antimicrobial susceptibility testing, compared to plate weight. **a** Meropenem (MEM) and minocycline (MH) ETEST MIC results. **b** MEM and MH disk diffusion results.

c MEM and MH ETEST MIC results on plates left on the bench top to dry. **d** MEM and MH disk diffusion results on plates left on the bench top to dry. **e** Ceftazidime (CAZ) ETEST MIC results. **f** CAZ disk diffusion results

(457.48 g/mol) or ceftazidime (546.58 g/mol). Because molecular weight correlates with diffusivity, these observations suggest that the higher the agar diffusion rate for an agent, the more susceptible is the resulting MIC and zone of inhibition value to agar depth.

Use of disk diffusion on solid medium to measure zone of inhibition remains a common AST tool. Past studies have identified that agar depth uniformity can affect the accuracy of this assay [7, 8], and CLSI guidelines call for agar to be at a depth of 4 mm. Without knowing how variances from this depth affect results, assay users do not know how rigorous they need to be when pouring plates. Here, we used MIC values obtained from ETESTs to quantify the relationship between agar depth and zoi. We find an inverse correlation between these two parameters and suggest that the smaller the agent under study, the greater is this correlation.

Acknowledgements This work was supported by NIH Grant (1R15HL126122-01) to T.R.S.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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