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NOTE

Better visualization and photodocumentation of zone of inhibition by staining cells and background agar differently

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Sensitivity or resistance of microorganisms to known or potential antimicrobial compounds is most often determined by the zone of inhibition test, also known as disk diffusion test or Kirby–Bauer test.[1,2] The test is widely used and helps physicians decide whether or not to prescribe a certain antibiotic for an infected patient. The procedure for performing the test has been standardized by the World Health Organization.[3] In brief, cells are spread on a plate and a small filter paper disk impregnated with the antibiotic is placed in the center of the plate. The absence of growth around the disk indicates sensitivity to the antibiotic. The diameter of the zone of inhibition is only a qualitative indicator of the potency of the drug as there are several other factors involved such as depth of the agar as well as the size and water solubility of the drug molecule. Nevertheless, the method is an easier and cheaper alternative than the broth dilution or the agar dilution methods. Characterization of new antibiotics usually includes a zone of inhibition result.[4]

Aggregatibacter actinomycetemcomitans, a naturally transformable,[5] Gram-negative facultative anaerobe, is a periodontal pathogen[6] that also belongs to the HACEK group of bacteria, which is known to cause endocarditis.[7] It has been observed in our laboratory that the zone of inhibition for *A. actinomycetemcomitans* has been difficult to detect because the colonies have a similar color as the background agar, resulting in the confluent colonies appearing transparent. Even if a faint zone of inhibition can be visualized by adjusting the intensity and angle of light source, photodocumentation of the faint zone of inhibition is very difficult. Other bacteria such as *E. coli* and *A. vinelandii* have also been reported to have transparent colonies and are difficult to photograph unless the incident light is carefully adjusted.[8] In this paper I show that this problem can be solved if the background agar and the microbial colonies can be stained differently.

Agar is a complex mixture of two carbohydrate polymers: agarose, a neutral linear polymer containing alternating units of β-1,3-linked-D-galactose and α-1,4-linked 3,6-anhydro-L-galactose; and agaropectin, an anionic polymer containing the same repeating unit as agarose except that some of the galactose units are modified with negatively charged side groups such as sulfate.[9] As agaropectin is negatively charged, it can be expected to bind cationic dyes such as crystal violet and methylene blue. Although, bacterial cell walls also bind cationic dyes, the stained cells can be expected to be differentiated from stained agar because of a metachromatic property of the dyes. Binding of crystal violet or methylene blue to anionic polysaccharides such as agaropectin results in a shift in λmax.[10] For example, λmax of crystal violet shifts from 585 to 510 nm. The shift is ascribed to 'stacking interactions' as excess dye molecules adsorb to dye molecules initially bound by electrostatic interactions.[11] Thus, I reasoned that staining of the agar plate with cationic dyes will stain the cells and the zone of inhibition differently.

I show here that by staining plates with crystal violet or methylene blue, the zone of inhibition can be not only more clearly visible but also easily photographed. Barely visible zone of inhibition is a common problem with many microorganisms. As examples I show here that great improvements in visibility can be achieved for the Gram-negative bacteria, *A. actinomycetemcomitans* (CU1000, a rough clinical isolate[12]), *Escherichia coli* (MV10) and *Proteus vulgaris* (ATCC 6380); and the Gram-positive bacteria, *Enterococcus faecalis* (ATCC 51299) as well as for yeast, *Saccharomyces cerevisiae* (baker's yeast, Fleischmann). The method can be applied universally for all microorganisms even if the zone of inhibition is already clearly visible. Gram-positive bacterium, *Staphylococcus aureus* (ATCC 25923) has been used as an example to demonstrate this.

The growth medium for *E. faecalis, S. aureus* and the *A. actinomycetemcomitans* contained tryptic soy broth ($25\,g\,l^{-1}$) and yeast extract ($6\,g\,l^{-1}$) without extra glucose added besides the $2.5\,g\,l^{-1}$ that is already present in tryptic soy.[13] *A. actinomycetemcomitans* was grown in 5% CO_2. *S. cerevisiae* was grown in medium containing 1.0% peptone and 4.0% glucose. *P. vulgaris* and *E. coli* were grown in Luria Bertani medium. All bacteria were grown at 37 °C except yeast, which was grown at 30 °C. All solid media contained 1.5% agar in addition to nutrients. All liquid and solid media were sterilized by the microwave method, which produces less Maillard reaction products, which are known to inhibit growth of some microorganisms, particularly, *A. actinomycetemcomitans.*[14,15] Unless otherwise mentioned, all plates

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contained 25 ml medium (4.4 mm). Approximately 10^7 cells were spread on plates and then a 7-mm diameter sterile filter paper disk containing an antibiotic was placed in the center of each plate. Antibiotics used were 120 μg kanamycin for *E. faecalis*, 60 μg kanamycin for *E. coli, P. vulgaris* and *S. aureus*, 30 μg kanamycin for *A. actinomycetemcomitans* and 60 μg clotrimazole for *S. cerevisiae*. Plates were incubated for 24 h, unless otherwise mentioned and then photographed using visible light from the bottom. The plates were then stained with 5 ml of either 0.2% crystal violet in 20% ethanol or 0.2% methylene blue in water for 30 s. This was then followed by five washes without shaking, each for ~1 min with 5 ml of 50% ethanol. The staining and destaining solutions were removed from the plates by aspiration using plastic transfer pipets. The plates were then photographed as described above.

Results of this new-modified Kirby–Bauer method are shown in Figure 1. Zones of inhibition are barely visible for *E. coli* and *P. vulgaris* and almost invisible for *A. actinomycetemcomitans* and *S. cerevisiae* (panels A and B). However, the zones are clearly visible after staining with either crystal violet or methylene blue (panels C and D). Methylene blue gives slightly better results than crystal violet. Results with a rough clinical isolate of *A. actinomycetemcomitans* (CU1000) are shown here. A commonly used smooth laboratory variant (Y4) of a clinical isolate is even more transparent and more difficult to visualize unless stained with crystal violet or methylene blue (data not shown). The zones of inhibition for the Gram-positive bacteria, *E. faecalis* and *S. aureus*, especially the latter are clearly visible even without staining. However, it is a good idea to apply the staining procedure for all bacteria, as the zone will be even more clearly visible and also some important features may be visible only after staining. This is seen for *S. aureus* after staining with methylene blue; a layer of yellow color is present at the boundary of the zone of inhibition (panels C and D). However, such extra ring is not clearly visible without staining the plate (panels A and B). The yellow color is indicative of a higher cell density as dense regions are destained more than isolated colonies. It has been reported before that there can be a greater cell density at the boundary of a zone of inhibition, which happens because of greater amount of nutrients available.[16] Similar to zone of inhibition studies, the Kirby–Bauer disk diffusion method can also be used to study growth promoting effect of some compounds.[17] Staining with methylene blue can probably be used to visualize regions of extra growth in such experiments.

Staining with either crystal violet or methylene blue greatly improves the sensitivity of the method such that the zone of inhibition can be detected after as early as 4 h of incubation (corresponding to <12 generations). At 5 h of incubation the zone is fully established and can be easily photographed (Figure 2) and thus, overnight incubation is not necessary. It is known that viewing or photographing

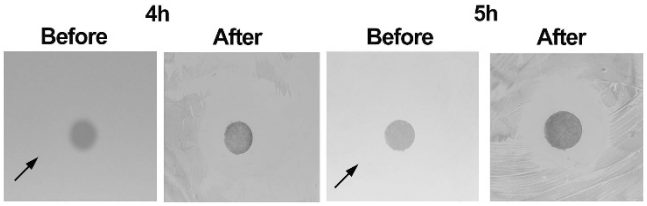

Figure 2 Early detection of zone of inhibition by staining procedure. Zones of inhibition for *E. coli* cells were photographed after 4 or 5 h of incubation and before and after staining with methylene blue. The boundaries of the zones of inhibition for the unstained plates are indicated with arrows. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

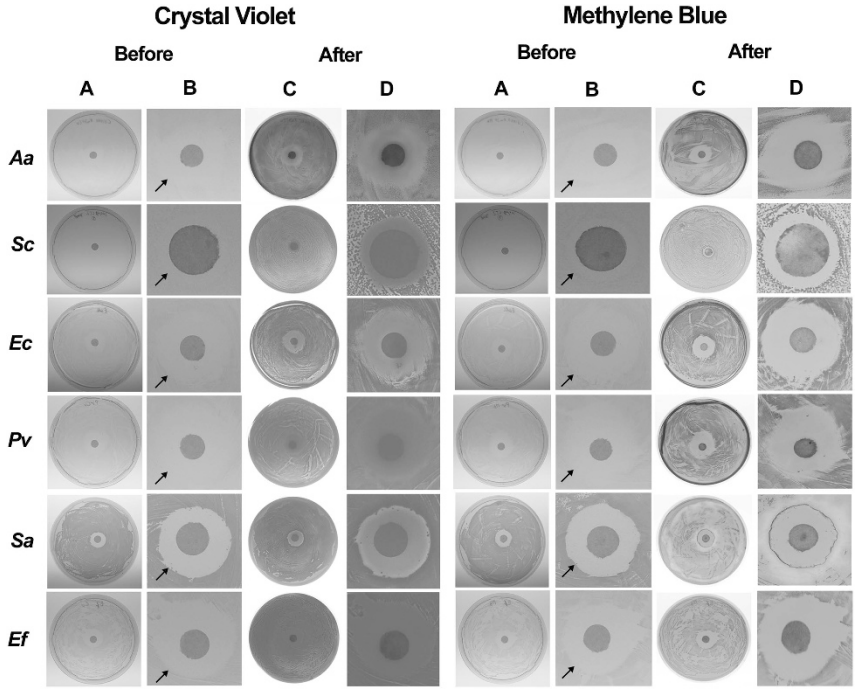

Figure 1 Visualization of zone of inhibition by staining with crystal violet and methylene blue. Images of plates before (A and B) and after (C and D) staining with crystal violet (left panels) or methylene blue (right panels). Pictures from A and C are enlarged in B and D to show the zone of inhibition more clearly. The boundaries of the zone of inhibition in panels B are indicated with arrows. *Aa (A. actinomycetemcomitans), Sc (S. cerevisiae), Ec (E. coli), Pv (P. vulgaris), Sa (S. aureus)* and *Ef (E. faecalis).* A full color version of this figure is available at *The Journal of Antibiotics* journal online.

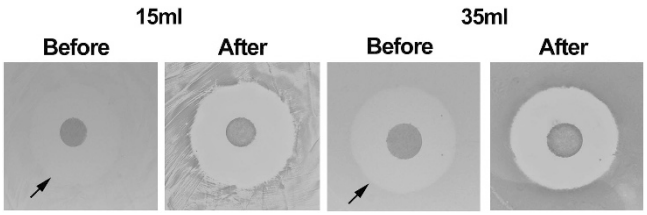

Figure 3 Effect of plate thickness on detection of zone of inhibition by staining procedure. *E. coli* cells were spread on thin (15 ml medium) or thick (35 ml medium) plates. Zones of inhibition were photographed after 24 h of incubation and before and after staining with methylene blue. The boundaries of the zones of inhibition for the unstained plates are indicated with arrows. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

a lawn of colonies on a plate is affected by the thickness of the plate. It is shown in Figure 3 that zone of inhibition can be clearly visualized and photographed both for a very thin (15 ml medium, 2.6 mm) or a very thick (35 ml medium, 6.2 mm) plate. Experiments for Figures 2 and 3 have been shown only for *E. coli*, the most widely studied bacterium and only staining with methylene blue has been shown but crystal violet is expected to give similar results.

Being a cationic dye, crystal violet can be used to stain negatively charged polymers such as DNA[18] and cell walls of bacteria, the latter of which is partially the basis of Gram staining to differentiate between Gram-positive and Gram-negative bacteria. It was shown that actually isolated cell wall material from Gram positive and Gram-negative bacteria binds crystal violet to the same extent.[19] The differentiation between the two types of bacteria is due to the subsequent steps of the Gram-staining procedure. In this report, I used only crystal violet and not the subsequent reagents of Gram-staining. This method can be used for both Gram-positive and Gram-negative bacteria. Destaining of cells was done with 50% ethanol. Even after five washes, more crystal violet can be washed out but that is not recommended because the background color of the agar will diminish. With further washings, the isolated colonies will remain colored but the dense region will be destained more (data not shown). On the other hand, methylene blue stain on background agar is very stable to further washings. If the filter paper disk comes off during destaining, it can be carefully placed back in the center or it may be completely removed; in case of the latter the region in the center will appear as unstained or less stained area (data not shown).

Addition of crystal violet or methylene blue to agar before spreading cells has been reported before but the purpose and methodology are different from what I report here. Crystal violet is often added to MacConkey agar at a concentration of 0.0001% (2000-fold less than that used here) to inhibit the growth of Gram-positive bacteria thereby selectively allowing Gram-negative bacteria to grow.[20] Addition of 0.00005% methylene blue (4000-fold less than what is used here) to agar plates allows zones of inhibition to be measured with greater precision.[21] However, this effect of methylene blue is unrelated to its dye property as it acts as an electron acceptor to give improved zone edge definition.[22]

The zone of inhibition method is qualitative and can only be used to classify the microbial strains as being susceptible, intermediate or resistant. The concentration of the antibiotic at the boundary of the zone of inhibition is the MIC of the antibiotic; however, it is not feasible to measure that concentration. A better and easier method for determining the MIC is the *E*-test in which a plastic strip containing a gradually decreasing concentration of an antibiotic is placed on an agar plate. Transparent and translucent colonies that are difficult to visualize and photograph will present a similar problem for the *E*-test and thus, the problem can also be expected to be solved by differentially staining agar and the cells with crystal violet or methylene blue. Observation of a zone of inhibition can also be used as a method for isolating new antibiotic-producing microorganisms.[23] It can be expected that staining with cationic dyes will also help in that procedure.

In conclusion, this method modifies the existing method to make the zone of inhibition more clearly visible and more easily photographed. Those bacteria for which the zone is already very clear, doing these extra steps that take <10 min will further improve the results. These few added steps may sometimes reveal some extra information. The staining method markedly improves the test such that the zone of inhibition can be clearly visible and documented after <5 h of incubation. Thus, overnight incubation is not necessary.

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