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Comparison of Monolayer and Bilayer Plates Used in Antibiotic Assay

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Standard curves of 5 antibiotics were determined in an antibiotic assay using bilayer and monolayer agar plates and AOAC-specified test organisms and agar media. *Micrococcus luteus* ATCC 9341a and antibiotic medium No. 2 were used to prepare the penicillin G standard curve. The same organism and antibiotic medium No. 11 were used to prepare the erythromycin standard curve. Standard curves for streptomycin, tetracycline, and gentamicin were prepared, respectively, with antibiotic medium No. 5 and *Bacillus subtilis* ATCC 6633, antibiotic medium No. 8 and *B. cereus* ATCC 11778, and antibiotic medium No. 11 and *Staphylococcus epidermidis* ATCC 12228. Assays of inhibition by meat fortified with penicillin, streptomycin, gentamicin, tetracycline, erythromycin also were performed on monolayer and bilayer plates. Differences in standard curves and inhibitory responses obtained with monolayer and bilayer plates were <10%. Thus, monolayer plates are acceptable for use in analyses of meat and poultry for antibiotics residues, with savings in laboratory resources and time.

The cylinder cup agar plate diffusion assay is used routinely for determining antibiotic potency (1). A dose line derived from the assay, known as the standard curve, shows the relationship between antibiotic concentration and zone of inhibition against a test microorganism. Among other factors, specificity and concentration of the test organism and volume of the assay agar affect the analysis.

The assay also has been used for determining antibiotic concentrations in animal feed (2–10). Traditionally, double or bilayer plates have been used. These consist of a base agar layer covered with another agar layer containing a specific number of organisms (1).

Monolayer plates used in antibiotic assay consist of a single agar layer containing a specific number of organisms. Use of monolayer plates requires less labor, materials, and time, and the analytical performance (sensitivity and accuracy) is equal to or better than with use of bilayer plates (11). Further, monolayer plates provide greater ease of use, better flexibility in obtaining optimal sensitivity, and improved standardization of the methodology (11). Use of monolayer plates would be acceptable provided the inhibitory zone of an antibiotic standard curve reference concentration on monolayer plates is within ±10% of the zone on bilayer plates (1).

The agar diffusion technique is used by the U.S. Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) for routine analysis of thousands of food animal tissue samples for antibiotic residues. The assay is also used to develop methods for detecting and identifying new antibiotics used in food animals. Use of bilayer plates is labor intensive and time consuming.

The present USDA/FSIS antibiotic assay procedure requires that a meat tissue be diluted 5-fold in 3 phosphate buffer solutions of pH 4.5, 6.0, and 8.0 (12). Therefore, assay plates should be sensitive enough to detect violative levels of antibiotic residues in the diluted tissue. The antibiotics and their violative concentrations are determined by the U.S. Food and Drug Administration and listed in the Federal Code of Regulations.

Use of monolayer plates reduces the time required to prepare plates and saves a significant amount of resources. However, such change should not interfere with the sensitivity of the assay for detecting violative levels of antibiotic residues in meat.

The study compares the performance of monolayer plates with bilayer plates on the basis of standard curve values of 5 antibiotics and inhibitory responses of antibiotic-fortified meat.

Experimental

Organisms

Test organisms were *Micrococcus luteus* (MLA) ATCC 9341a, *Bacillus subtilis* (BS) ATCC 6633, *B.*

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cereus (BC) ATCC 11778, and *Staphylococcus epidermidis* (SE) ATCC 12228. Spores of BS, catalogue No. 0453-60, and BC, catalogue No. 0959-52, were obtained from Difco, Detroit, MI. Cultures of SE and MLA were cryopreserved in methylcellulose (13).

Media and General Preparation of Plates

Antibiotic medium No. 2 (catalogue No. 10912), No. 5 (catalogue No. 10953), and No. 8 (catalogue No. 10965) were prepared according to manufacturer's (Beckton and Dickinson, Cockeysville, MD) directions.

Bilayer plates were prepared by pipetting 10 mL agar into each plate. After the agar had solidified, the entire surface was covered with 4.0 mL of the same agar seeded with a known number of organisms.

Monolayer plates were prepared by pouring into each plate 8.0 mL test agar seeded with a known number of test organism.

Antibiotic Standards

Penicillin G (catalogue No. P-7794), streptomycin sulfate (catalogue No. S-6501), erythromycin (catalogue No. E-6376), tetracycline (catalogue No. T-3383), and gentamicin (catalogue No. G-3632) were obtained from Sigma Chemical Co., St. Louis, MO.

Preparation of Specific Plates

(a) *BC plates.*—A 0.15 mL portion of BC spore suspension (2.5×10^7 spores/mL) was added to 100 mL antibiotic medium No. 8 kept in a 48°C water bath and mixed gently. After the 45 min incubation required to germinate BC spores before plates are prepared, 8.0 mL agar was pipetted into each plate (100×15 mm) for a monolayer plate. For a bilayer plate, 4.0 mL was pipetted over a 10.0 mL base layer of antibiotic medium No. 8. Plates were refrigerated and discarded after 5 days.

(b) *BS plates.*—Monolayer and bilayer plates were prepared as described above, except 100 mL antibiotic medium No. 5 was seeded with 0.5 mL BS spore suspension (2.5×10^7 spores/mL) and incubated for 75 min in a 48°C water bath to allow BS spores to germinate before plates are prepared.

(c) *MLA-2 plates.*—Plates were prepared as described above, except 100 mL antibiotic medium No. 2 was seeded with 0.15 mL MLA (1×10^8/mL) with no preincubation in the water bath.

(d) *MLA-11 plates.*—Plates were prepared as described in **(c)** above, except agar was replaced with antibiotic medium No. 11.

(e) *SE plates.*—Plates were prepared as described in **(d)** above, except antibiotic medium No. 11 was seeded with 0.15 mL SE (1×10^8/mL).

Preparation of Antibiotic Standards

AOAC-recommended buffers were used for preparing standard solutions of each of the test antibiotics (14).

A stock solution (1000 μg/mL) of penicillin G was made in pH 6.0 phosphate buffer. Working standards (0.0125, 0.025, 0.05, 0.1, and 0.2 μg/mL), also in pH 6.0 phosphate buffer, were prepared from stock solution.

Similarly, working standards of gentamicin sulfate (0.08, 0.16, 0.32, 0.64, and 1.28 μg/mL) and streptomycin sulfate (0.125, 0.25, 0.5, 1.0, and 2.0 μg/mL) were prepared from stock solution in pH 8.0 phosphate buffer. Working standards of erythromycin sulfate (0.05, 0.1, 0.2, 0.4, and 0.8 μg/mL) prepared in pH 8.0 buffer from a stock solution (1000 μg/mL) were made by dissolving the salt in 2 mL methanol and then diluting with pH 8.0 phosphate buffer to a final concentration of 1000 μg/mL. Tetracycline (0.08, 0.16, 0.32, 0.64, and 1.28 μg/mL) working standards were prepared from stock solution (1000 μg/mL), which was prepared by dissolving the salt in 2 mL 1N HCl and diluting with pH 4.5 phosphate buffer to a final concentration of 1000 μg/mL.

The first and the third working solutions of all antibiotics were used as the minimum inhibitory and the reference concentrations, respectively.

Determination of Standard Curves

Stainless steel bioassay spiders with 6 wells (Arthur E. Farmer, Trenton, NJ) were placed on each of 12 monolayer and bilayer plates containing antibiotic agar No. 2. Two hundred microliters of one concentration of working penicillin G solution was pipetted into 3 alternate wells of spiders. In the 3 other wells, 200 μL reference (i.e., the third concentration of the standard solution) was pipetted. Similarly, other plates were inoculated with other concentrations of standards and reference solutions. After incubation at 37°C for 18 h, spiders were removed and the zone of inhibition on each plate was recorded. Standard graphs from monolayer and bilayer plate data were prepared with an FSIS-developed computer program based on AOAC standard-curve methodology (15).

Similarly, standard graphs of monolayer and bilayer plate data for streptomycin, tetracycline, gentamicin, and erythromycin were generated by computer.

Inhibition of Test Microorganisms by Animal Tissue Fortified with Antibiotic

As described in the FSIS laboratory guidebook (12), muscle tissue fortified with a known quantity of penicillin was extracted in pH 6.0 phosphate buffer. By using steel spiders, a set of monolayer and bilayer plates seeded with MLA were inoculated with the meat extract. After plates were incubated at 37°C for 18 h, zones of inhibition produced on both plate types were recorded. Similarly, zones of inhibition on both plate

types caused by extracts of meat fortified with other antibiotics were recorded.

Results

Computer-generated values for y intercepts, slopes, and mean references for 5 antibiotic standard curves for monolayer and bilayer plate data are presented in Table 1.

Bilayer plate data gave higher values of the logarithm of the y intercept for all antibiotics except streptomycin and erythromycin than values from monolayer plate data. Slopes for monolayer and bilayer plate data for all antibiotics differed by ± 0.1, except for penicillin and tetracycline. The difference in the mean reference values for monolayer and bilayer plate data for all antibiotics was highest for penicillin G (9.7%) and lowest for streptomycin (2.3%).

The difference in standard curve values indicates that the responses of monolayer plates, as judged from the zones of inhibition by concentrations of test antibiotics, were different from those of bilayer plates. However, on the basis of mean values of reference zones, the differences in the responses monolayer and bilayer plates were within the acceptable 10% limit.

By using the same program (15), standard curves of monolayer and bilayer plate data for all antibiotics were computer generated, an example of which is presented in Figure 1. Standard curve data for all other antibiotics were similar. Zones of inhibition produced by extracts of antibiotic-fortified muscle tissues on monolayer and bilayer plates are presented in Table 2. The average difference in the zones of inhibition on monolayer and bilayer plates for the antibiotics ranged from 0.2 to 0.5 mm. Except for streptomycin, zone sizes for all antibiotics were higher with monolayer plates than with bilayer plates.

Statistical Analysis of Data

Mean reference values (Table 1) were analyzed further by the paired *t*-test to determine if differences were significant (16). With 8 degrees of freedom, the critical value for a 2-tailed test was ± 2.306 at the 0.05 significance level. The *t* values were calculated on the basis of mean difference between the paired values and their standard deviation (Table 3). Values for streptomycin (1.45) and erythromycin (2.01) indicate that the differences between monolayer and bilayer values for the 2 antibiotics were not significant at the 5% level. However, *t* values of −7.70 for penicillin G, 5.15 for tetracycline, and −2.96 for gentamicin indicate that the differences between monolayer and bilayer values were significant at the 5% level. Values for penicillin and gentamicin indicate that monolayer plates are more sensitive to these antibiotics whereas bilayer plates are more sensitive to tetracycline. However, the differences in mean reference values (Table 1) indicates that the differences noted (Table 3) between monolayer and bilayer plate values do not differ by more than 10%.

Zones of inhibition produced by muscle tissue extracts fortified with various antibiotics on monolayer and bilayer plates were analyzed statistically. Differences in inhibition were not significant at the 5% level (Table 2).

Discussion

The agar diffusion plate assay for antibiotics is usually performed with plates containing 2 layers of agar. Among many factors, the amount of agar affects the sensitivity of the assay. Thus, a specific amount of agar is recommended to achieve the desired level of assay sensitivity (17).

The main purpose of the study was to determine if bilayer plates could be replaced with monolayer plates. If so, then monolayer plates, which require less time and material and are easy to prepare, could be used by USDA/FSIS laboratories without compromising the efficacy of the assay used to monitor antibiotic residues in meat and poultry. As the agency checks thousands of meat samples for antibiotic residues annually, use of monolayer plates would save enormous amount of time and resources.

The AOAC standard-curve assay procedure was chosen to avoid discrepancy arising from methodology for comparing assay responses with monolayer and bilayer plates. The study shows that the response of monolayer plates to streptomycin and erythromycin does not differ significantly from that of the bilayer plates at the 5% level. Although the response of mono-

Table 1. Logarithm of y intercept, slope, and mean reference values of standard curves for various antibiotics obtained with bilayer (BL) and monolayer (ML) plates

	Log of y intercept		Slope		Mean reference		
Antibiotic	BL	ML	BL	ML	BL	ML	Difference, %
Streptomycin	−2.2430	−1.9518	0.1048	0.0948	17.6	17.2	2.3
Penicillin G	−2.9804	−3.6039	0.0795	0.1010	17.5	19.2	9.7
Tetracycline	−1.9920	−2.0215	0.0788	0.0878	19.5	17.9	8.2
Gentamicin	−2.2223	−2.2275	0.1053	0.1001	17.0	18.0	5.9
Erythromycin	−2.6778	−2.6286	0.1088	0.1123	18.5	17.7	4.3

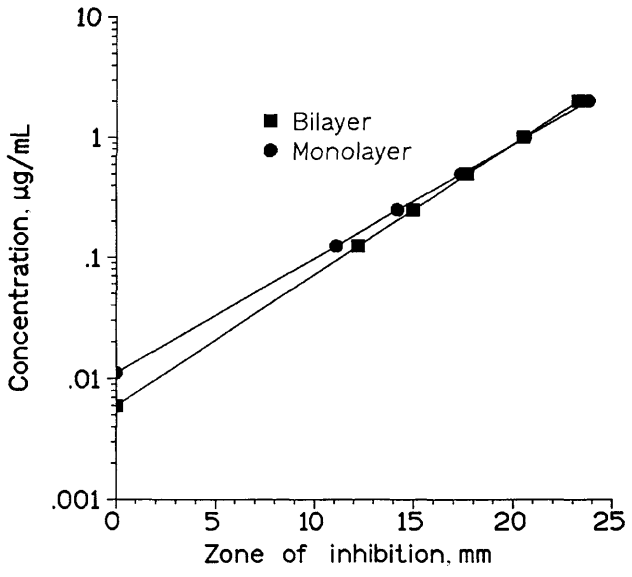

Figure 1. Standard curves for streptomycin.

layer plates to penicillin, tetracycline, and gentamicin vary from that of bilayer plates by more than 5%, the differences are within 10%. This change in response was expected because the volume of agar directly affects the diffusion of antibiotic.

This study and earlier pilot studies show that the performance of monolayer plates, like bilayer plates, varies with agar smoothness, uniformity, and depth. Performance does not change for 5 days if plates are sleeved and stored at 4°C. However, for optimum performance, freshly prepared plates should be tested with reference concentrations of antibiotics before use in analysis. Although, 8.0 mL agar used in monolayer plates provides adequate support for spiders, careless placing can tear the agar surface.

The increased sensitivity of monolayer plates for some antibiotics was another positive outcome. This higher sensitivity, resulting from the quantity of agar used in monolayer plates, allows federal laboratories to detect antibiotics, usually in microgram quantities, as violative levels set by the Federal Code of Regulations. Additionally, it has been reported that assay plates easily can be adjusted by manipulating the concentration of organisms in the agar (11, 17). Thus, USDA/FSIS laboratories have been able to adjust assay sensitivity by regulating the sensitivity of plates. The simple adjustment has helped USDA/FSIS develop methods for detecting violative levels of practically all antibiotics used in food animals.

Table 2. Zone of inhibition by antibiotics in muscle tissue on monolayer and bilayer plates

	Mean zone of inhibition, mm (n = 9)	
Antibiotic	Monolayer plate	Bilayer plate
Penicillin	15.5	15.0
Streptomycin	10.0	10.2
Erythromycin	16.5	16.0
Tetracycline	11.8	11.4
Gentamicin	12.5	12.0

Table 3. Statistical analysis of monolayer and bilayer reference values of antibiotic curves

	Differences		
Antibiotic	Mean	Standard deviation	t value
Streptomycin	0.38	0.806	1.45
Penicillin G	−1.71	0.666	−7.70
Tetracycline	1.58	0.918	5.15
Gentamicin	1.00	1.012	−2.96
Erythromycin	0.83	1.240	2.01

Since 1992, monolayer plates have been used routinely in all USDA/FSIS laboratories for analysis of antibiotic residues. Adaptation of monolayer plates has saved resources and has improved the analytical capabilities of FSIS in antibiotic residue detection.

Conclusion

The study suggests that antibiotic standard curves derived from use of monolayer and bilayer plates are not significantly different. The study also indicates that inhibition of test organism in agar cup diffusion technique by the antibiotic residue in meat tissue on monolayer plates is not significantly different from those observed on bilayer plates. The data indicate that monolayer plates are acceptable for use in analyses of meat and poultry tissues for antibiotic residues.

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