Effect of Plate Preincubation on *Bacillus stearothermophilus* Disc Assay Zone Diameters

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ABSTRACT

Effect of plate preincubation on the *Bacillus stearothermophilus* disc assay zone size was studied. Each of three raw milk samples were subdivided and treated with five levels of US Pharmacopeial reference standard penicillin G. Freshly prepared, 2.5- and 5-d old seeded disc assay culture plates were preincubated at 64°C for 0 (control), 20, 40, 60, 80, 100, and 120 min prior to placement of discs on the medium surface.

Plate age did not have a significant effect on the size of the zone diameter. Following 20 and 40 min of plate preincubation, zone diameters were in some, but not all, cases significantly different from the control. Plate preincubation of 60 min or greater always significantly decreased zone diameter. Routine preincubation of *B. stearothermophilus* plates is not recommended. In situations where time is a critical factor, plates should be preincubated no longer than 40 min.

INTRODUCTION

Antibiotic residues adulterate raw milk. With few exceptions, their presence in raw milk has been attributed to poor farm management practices (7). Problems created by the presence of antibiotic residues in milk and milk products are well-documented and have been reviewed (9). Considerable testing by processors and regulatory agencies is done to detect and minimize acceptance of antibiotic-contaminated milk and to assure consumers that milk and other dairy foods contaminated with antibiotics are not marketed. Antibiotic screening of all incoming milk shipments is at most plants a routine quality check prior to unloading the milk. For this reason, several rapid antibiotic screening tests have been developed and marketed (4, 5, 14).

In some situations, the use of rapid antibiotic screening tests is neither feasible nor economical, and processors have relied on the qualitative *B. stearothermophilus* disc assay (10). The disc assay represents a well-accepted method for determining the presence of antibiotics in raw milk. The procedure has been standardized, adopted by the National Conference on Interstate Milk Shipments (1), and is cited in the Grade A Pasteurized Milk Ordinance. A significant drawback of this test is the minimum 2.5-h incubation period.

Several modifications and adaptations of the *B. stearothermophilus* disc assay have been reported. Balbi and Hartman (2) increased the sensitivity of the disc assay from .005 to .003 IU penicillin-G/ml by adding a known quantity of the antibiotic to the assay discs. Procedural modifications of Ginn et al. (6) permit quantitative estimates of β-lactam residues in raw milk at levels >.016 IU penicillin G/ml. Rajkowski and Messer (12) developed a qualitative *B. stearothermophilus* disc assay procedure for screening casein and caseinate products. Rajkowski et al. (11) studied disc assay detectability levels of four β-lactam antibiotics in eight milk products.

A modification of the *B. stearothermophilus* disc assay procedure that is sometimes implemented in processing plant quality control laboratories is the preincubation of disc assay plates prior to disc addition. The acceptability of this practice has not been documented. Thus, the purpose of this study was to determine if plate preincubation affects *B. stearothermophilus* disc assay zone size.
MATERIALS AND METHODS

**Milk Samples**

Three raw milk samples were obtained from the Louisiana State University dairy farm. The samples were collected in sterile 1-L glass containers and transported (at or below 4.4°C) to the dairy testing laboratory where they were stored at 4.4°C.

Penicillin-treated subsamples of each milk sample were prepared within 24 h of milk sample collection. Penicillin G stock solution was made as described previously (15). One vial of frozen penicillin G stock solution (1.25 mg/ml; 2000 IU/ml) was thawed and a 1-ml aliquot transferred to a volumetric flask and diluted to 25 ml with 1% phosphate buffer (6). The standard was further diluted by transfer of aliquots of .5, 1.0, 2.0, and 4.0 ml to volumetric flasks and contents of each flask diluted to 100 ml with phosphate buffer. From each flask, 1-ml aliquots were transferred to separate 100-ml volumetric flasks and brought to volume with a portion of a raw milk sample. The four treated subsamples and an untreated subsample were transferred to glass beakers and held at 4.4°C prior to antibiotic testing.

**Bacillus stearothermophilus Disc Assay**

Treated and untreated milk samples were assayed for penicillin G residues by a modified *B. stearothermophilus* disc assay procedure (15). Preparation of the assay plates was scheduled such that freshly prepared, 2.5- and 5-d old plates were available for antibiotic screening. To determine the effect of preincubation on zone diameter size, freshly prepared, 2.5- and 5-d old plates were preincubated at 64 ± 2°C for 0, 20, 40, 60, 80, 100, and 120 min prior to discs being placed on the medium surface. Six replicate discs were prepared for each milk sample by penicillin concentration by plate age by preincubation time combination. Assay plates were stacked no more than two high in a continuous flow, water-jacketed incubator to ensure rapid and even heating of the growth medium. Following disc addition, all plates were returned to the incubator until well-defined zones of inhibition were observed. Zone diameters were measured to the nearest .1 mm using vernier calipers.

**Statistical Analysis**

Least squares analysis of variance using factorial fixed effects models and pairwise contrasts of selected means were used for analysis and interpretation of results. Calculations were performed using SAS Version 5.15 (16) on an IBM Model 3084 computer. Model 1 included main effects of replication, preincubation time, plate age, antibiotic level, and all two-way interactions except those involving replication. Model 1 was for initial determination of significant overall sources of variation. Because the main effect of age and two-way interactions involving age were non-significant, they were dropped from Model 1, resulting in Model 2. The reason for dropping these sources from the model was to restore degrees of freedom to the error term, since it was obvious from Model 1 that plate age was not involved in determination of zone diameter. Reduction in $R^2$ from Model 1 to Model 2 was .005%.

**RESULTS AND DISCUSSION**

Precision of the disc assay procedure, defined as the pooled standard error (17), ranged from .26 to .91, with an average precision among nine laboratories participating in a collaborative study of .45 (6). *B. stearothermophilus* disc assay precision in our laboratory for this study was .34.

Statistical analysis of the zone diameter data indicated that replication, length of preincubation, penicillin G concentration, and penicillin G concentration by length of preincubation interaction were significant ($P<.01$) sources of zone diameter variation (Table 1). The significant interaction indicates that changes in zone diameters did not vary uniformly among penicillin G concentrations over the different plate preincubation times. Age of the plates did not have a significant effect on zone diameter. This observation supports current recommendations (13) that *B. stearothermophilus* disc assay plates may be used for up to 5 d after their initial preparation.

Disc assay analyses on treated and untreated milk samples using plates receiving no preincubation most closely follows recommended testing procedures. Thus, zone diameters on plates receiving no preincubation were used as
TABLE 1. Results of least squares analyses of variance on *Baeillus stearothermophilus* disc assay zone diameters.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of squares</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Model 1</td>
</tr>
<tr>
<td>Replication (R)</td>
<td>2</td>
<td>1472.04**</td>
</tr>
<tr>
<td>Preincubation (PI)</td>
<td>6</td>
<td>19141.90**</td>
</tr>
<tr>
<td>Plate age (A)</td>
<td>2</td>
<td>2.97</td>
</tr>
<tr>
<td>Antibiotic level (AB)</td>
<td>4</td>
<td>93136.37**</td>
</tr>
<tr>
<td>PI X A</td>
<td>12</td>
<td>.83</td>
</tr>
<tr>
<td>PI X AB</td>
<td>24</td>
<td>12042.08**</td>
</tr>
<tr>
<td>A X AB</td>
<td>8</td>
<td>3.00</td>
</tr>
<tr>
<td>Residual</td>
<td>18</td>
<td>9432.16</td>
</tr>
</tbody>
</table>

1 Not applicable.

**P (Type I error)<.01.

the control or standard against which all other means were compared. Significant differences were determined by contrasting least squares means (Table 2). At a penicillin G concentration of 5.0 ng/ml, zone diameters on plates preincubated for 20 min were smaller ($P<.05$) than zone diameters on control plates. At a penicillin G concentration of 10.0 ng/ml, zone diameters on plates preincubated for 20 and 40 min were smaller ($P<.05$) than zone diameters for control plates. Except for untreated milk samples, disc assay zone diameters decreased significantly ($P<.05$) when plates were preincubated for 60 min or longer.

A significant decrease in *B. stearothermophilus* disc assay zone diameters following plate preincubation of $>60$ min may be attributed to a combination of changes in diffusion medium inoculum density and extent of antibiotic diffusion. Because the amount of time that the discs are in contact with the medium decreased, the distance that penicillin molecules diffuse from the discs may be limited. Also, during preincubation, *B. stearothermophilus* cells multiply and the cell density of the diffusion medium increases. An increase in cell density increases the number of potential sites for cell-wall binding, which may, in effect, limit diffusion of the antibiotic molecules and result in a reduced zone diameter. This hypothesis is supported by previous research (3, 8), which demonstrated that as inoculum concentration in the diffusion medium increased, sizes of the zones of inhibition decreased.

TABLE 2. Least squares means of *Bacillus stearothermophilus* disc assay zone diameters produced by five different penicillin G concentrations after seven preincubation times.

<table>
<thead>
<tr>
<th>Penicillin G (ng/ml)</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(zone diameter in mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>12.7a</td>
<td>12.7a</td>
<td>12.7a</td>
<td>12.7a</td>
<td>12.7a</td>
<td>12.7a</td>
<td>12.7a</td>
</tr>
<tr>
<td>2.5</td>
<td>13.6a</td>
<td>12.4ab</td>
<td>13.6a</td>
<td>13.2b</td>
<td>12.7c</td>
<td>12.7c</td>
<td>12.7c</td>
</tr>
<tr>
<td>5.0</td>
<td>16.4a</td>
<td>16.1b</td>
<td>16.2b</td>
<td>15.5c</td>
<td>14.9d</td>
<td>13.2e</td>
<td>13.2f</td>
</tr>
<tr>
<td>10.0</td>
<td>19.3a</td>
<td>19.8b</td>
<td>19.1b</td>
<td>18.4c</td>
<td>17.3d</td>
<td>15.7e</td>
<td>15.9f</td>
</tr>
<tr>
<td>20.1</td>
<td>21.6a</td>
<td>21.5a</td>
<td>21.6a</td>
<td>20.7b</td>
<td>19.6c</td>
<td>18.0d</td>
<td>15.6e</td>
</tr>
</tbody>
</table>

a,b,c,d,e,f Means (n = 54) in the same row with different superscripts differ significantly ($P<.05$).

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CONCLUSIONS

Economics, practicality, and a desire to use the same test, and hopefully obtain the same results as regulatory agencies are some reasons for selection of disc assay screening procedure. The recommended procedure for conducting the disc assay does not include a period of plate preincubation. Results from this study indicate that when plate preincubation is used as to shorten B. stearothermophilus disc assay, zone diameter for milk samples containing antibiotics can be reduced. Routine preincubation of B. stearothermophilus plates is therefore not recommended. In situations where time is critical, plates should be preincubated no longer than 40 min. Considering the potential public health risks and financial losses that can occur if one or more silos of milk are contaminated with antibiotic-adultered milk, all milk shipments producing a zone of inhibition on a preincubated disc assay plate should be isolated and analyzed by official testing procedures.

REFERENCES