Detection of Penicillin G in Milk Using a Conductimetric Method

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ABSTRACT

A highly sensitive method was developed that used conductance measurement for the detection of penicillin G in milk. The method is based on the inhibition by the antibiotic on the growth of *Bacillus stearothermophilus* ATCC 10149. The conductance change in PM indicator agar containing the bacterial spores was continuously monitored at 55°C by a microbiological analyzer, and the detection time was delayed when penicillin G was present in the samples. The detection limit of the method for penicillin G was 0.00016 IU/ml with a detection time of about 3.4 h, but only a narrow range (.00016 to .00062 IU/ml) of the antibiotic could be quantitatively analyzed. The conductimetric method is about 30 times more sensitive than several methods currently used. In addition, the conductance measurement is fully automatic, and multiple samples, 120 or 240, can be analyzed simultaneously.

(Key words: milk, penicillin G, conductance, *Bacillus stearothermophilus*)

Abbreviation key: DT = detection time.

INTRODUCTION

Antibiotics have been used as a part of dairy cattle management for several decades. Unfortunately, antibiotics and drugs used in treatments may enter the milk supply. Once antibiotics are present in milk, they are difficult to eliminate, and the presence of these drugs is of public health concern. An estimated 5 to 10% of American adults are hypersensitive to antibiotics (10). After ingestion, as little as .003 IU of penicillin G may cause allergic responses (14). The antibiotic residues also may create an environment that is favorable for resistant bacterial strains.

The presence of antibiotics in milk has also created problems in the dairy industry, including inadequate curdling of milk, improper ripening of cheeses, and decreased acid or flavor production (2, 3). A 50% inhibition of cheese and yogurt starter cultures by .2 IU/ml of penicillin was reported (6). The FDA considers antibiotic-contaminated milk to be adulterated. Therefore, the test of antibiotics in milk is important for the dairy industry. The sensitivities of several currently used methods for the detection of penicillin G in milk are the *Sarcinia lutea* cylinder-plate method, .02 IU/ml; Penzyme test, .01 IU/ml; *Bacillus stearothermophilus* disc assay, .005 IU/ml; Charm test, .005 IU/ml; and Delvotest-P, .005 IU/ml (1, 2, 16). For the detection of trace residues (<0.005 IU/ml) of penicillin G in milk, a more sensitive, and preferably automatic, method is needed.

The changes in electrical properties of the culture media have been utilized for the rapid estimation of total, mesophilic, and psychrotrophic counts (9), coliforms (8), salmonella (4, 11), abnormal milk (12), and bacteriophage in Cheddar cheese making (17). Electrical change was also used as a growth index of lactic acid bacteria in milk (13).

Because of the susceptibility of *B. stearothermophilus* to penicillin G, monitoring of changes in conductance may be an efficient way for the detection of the antibiotic in milk. Previously, Okigbo and Richardson (15) reported an impedance method using the Bactometer (bioMerieux Vitel, Inc., Hazelwood, MS) to detect low concentrations of penicillin G in sterile milk inoculated with 5% active lactic culture. Although the test is sensitive (.001 IU/ml), quantitative results were not obtained, and a long incubation time (5 to 10 h) was needed. The purpose of our study was to develop a sensitive and quantitative conducti-
metric assay for detecting penicillin G in milk using a Malthus 2000 microbiological analyzer (Malthus Instruments Limited, Crawley, England) with \( B. \) *stearothermophilus* as the test organism.

**MATERIALS AND METHODS**

**Media and Culture**

Trypticase soy agar and trypticase soy broth were from BBL (Becton Dickinson, Cockeysville, MD). The medium used for total viable count (SPYE broth; catalog number 4W001) was a product of Malthus Instruments. *Bacillus stearothermophilus* ATCC 10149 spore suspension (catalog number 1802-33-9), Bacto-PM positive control (nonfat dry milk containing .12 IU of potassium penicillin; catalog number 1802-33-9), Bacto-PM negative control (inhibitor-free nonfat dry milk; catalog number 1803-63-1), and Bacto-PM indicator agar (catalog number 1800-15-3) were obtained from Difco Laboratories (Detroit, MI). Penicillinase (EC 3.5.2.6; catalog number P-0389) was a product of Sigma Chemicals (St. Louis, MO).

**Optimization of Conductimetric Method**

Conductance changes, measured in microsiemens, were automatically monitored and graphically represented by the software package of the Malthus 2000 system. To obtain a conductance curve of good quality (i.e., a curve having a stable baseline, a sharp slope, and a high conductance value), four different media (trypticase soy broth, trypticase soy agar, SPYE broth, and Bacto-PM indicator agar) were used for the inoculation of *B. stearothermophilus* spores. The media were autoclaved at 121°C for 15 min and seeded with spores to give a concentration of 1% (1 ml of stock spore suspension added to 100 ml of sterile medium; final concentration about \( 10^6 \) spores/ml). The media containing the spores were dispensed in 2-ml amounts in 5-ml capacity conductance cells and then incubated at 55°C in the water bath of the Malthus 2000 microbiological analyzer. Readings of conductance for each cell were taken every 6 min over 24 h. Results were obtained numerically as conductance data or graphically as conductance growth curves. Detection time (DT) in hours for each cell was automatically determined by the instrument software when conductance values increased by 1 \( \mu S \) or more for three consecutive scans.

**Construction of Calibration Curve of Penicillin G**

The positive control (Difco) medium, containing .12 IU of penicillin G, was serially diluted twofold with the PM negative control medium to obtain samples containing decreasing concentrations of the antibiotic. Following the addition of 1 ml of each diluted positive control into the conductance cell (six replicates), 2 ml of 50°C PM indicator agar containing 1% spore suspension were added to each cell, mixed thoroughly, and incubated at 55°C for the monitoring of conductance change. The sensitivity of the conductimetric assay was defined as the concentration of penicillin G that had a DT value higher than the negative control at a significance level of .01, using the \( t \) test.

**Detection of Penicillin G in Milk Powders and Infant Formulas**

Twelve milk products imported into this country, including 6 milk powders and 6 infant formulas, were reconstituted (10 g added to 90 ml of deionized water) and serially diluted twofold with PM negative control. Each diluted sample was subdivided into two portions; one portion (1 ml) was heated at 82°C for 3 min, cooled immediately in an ice bath, and treated with .1 ml of \( \beta \)-lactamase (165 U/ml) at 37°C for 30 min. Following the addition of 1 ml of each sample (positive and negative controls and enzyme-treated and untreated samples) into the conductance cell, 2 ml of PM indicator agar containing 1% *B. stearothermophilus* spore were added and mixed thoroughly. Cells were incubated at 55°C for the measurement of conductance change. Penicillin G in the milk products was also determined by the *B. stearothermophilus* disc assay (1).

**RESULTS AND DISCUSSION**

**Optimal Condition for Conductance Measurement**

Figure 1 shows curves of conductance change using four different media inoculated...
DETECTION OF PENICILLIN G IN MILK

4.5
8.0
12.0
Time (h)

Figure 1. Comparison of conductance curves using four different media containing 1% (10^6 spores/ml) Bacillus stearothermophilus spores. A. PM indicator agar; B. trypticase soy broth; C. tryptic soy agar; and D. SPYE broth. The letters a, b, and c are the detection times of curves A, B, and C, respectively. Curve D does not have a measurable detection time.

with B. stearothermophilus spores at a concentration of 1% (10^6 spores/ml). A successful conductimetric method depends on the quality of curves, which are influenced by the specific combinations of sample and medium. A conductance curve of good quality should exhibit a stable baseline, followed by a sharp accelerating slope and a high peak value. Because some metabolite end products yield a stronger conductance signal, the selection of an appropriate medium is crucial. As illustrated in Figure 1, PM indicator agar met the criteria for a good quality curve. In addition, the amplitude of conductance generated by PM indicator agar was at least twice those obtained by the other three media. Therefore, PM indicator agar was selected as the medium for further tests.

Although 1% (about 10^6 spores/ml) of spore suspension (Difco) is generally recommended for the B. stearothermophilus disc assay, this concentration might not be suitable for the conductance methodology. Three inoculum percentage (1, .1, and .01%) were used in the PM indicator agar to evaluate the influence of inoculum on the conductance curves. As shown in Figure 2, spore concentration had little effect on the curve quality except that the DT was inversely proportional to the spore concentrations used. The DT were 3.3, 3.9, and 4.2 h for spore concentrations of 1, .1, and .01%, respectively. However, the three conductance curves showed the same trend of conductance change and had similar peak conductance, regardless of the initial spore load. Because time is an important factor for the quality control of milk, 1% spore concentrations were used in the following experiments.

Sensitivity and Effect of Penicillin G on Conductance Curve

The effect of penicillin G on the conductance curves is illustrated in Figure 3. Obviously, the higher the concentration of penicillin G, the longer was the DT. The negative control had a DT of 3.1 h. As the concentration of the antibiotic increased, the DT was higher, and the slope of the curve was smaller. When the concentration of penicillin G was ≥ .0025 IU/ml (Figure 3; curves G and H), the inhibition of the growth of B. stearothermophilus was so strong that a level curve was obtained. Under this condition, no DT could be obtained during a 24-h incubation period. At a penicillin G concentration of .0012 IU/ml (Figure 3; curve F), the slope of the curve was so small that a correct DT was difficult to assign. Inevitably, the DT of the negative and positive controls may be subject to change because the physiological state and the concentration of the spore suspension are

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Figure 3. Conductance curves of reconstituted nonfat dry milk samples containing different concentrations of penicillin G: A, negative control (inhibitor free); B, .00008 IU/ml; C, .00015 IU/ml; D, .00031 IU/ml; E, .00062 IU/ml; F, .00125 IU/ml; G, .0025 IU/ml; and H, .005 IU/ml. The letters a, b, c, d, e, and f are the detection times of curves A, B, C, D, E, and F, respectively.

difficult to maintain at fixed values between lots and between experiments. However, in many separate experiments, the DT of the negative control was always between 2.6 and 3.2 h.

In testing serially diluted positive samples, the DT of samples containing penicillin G higher than .005 IU/ml could not be completely restored to the value of negative control by penicillinase (final concentration 15 U/ml) treatment at 37°C for 30 min. This result may have been due to the incomplete digestion of penicillin G by the enzyme under the conditions used. Complete reversal was achieved by proper dilution of samples with penicillin G concentrations >.005 IU/ml.

The sensitivity of the conductimetric assay for penicillin G was .00016 IU/ml; at this concentration the DT (mean of six replicates) was 3.38 h, which was significantly higher (P = .01) than that of the negative control (DT 3.11 h). This level was about 30 times more sensitive than the B. stearothermophilus disc assay, Charm test, and the Delvotest-P, which have detection limits around .005 IU/ml of milk (1, 16). The conductimetric assay also has a potential to be used as a rapid screen test by setting an appropriate DT for the negative specimens; samples with DT higher than the preset DT would be considered as inhibitor-positive. However, because of the experimental setting such a DT threshold.

Linear Relationship Between DT and Penicillin G

A linear regression line (r = .99) correlating the DT (in hours) and the concentration (international units per milliliter) of penicillin G is shown in Figure 4. The range suitable for quantitative determination of penicillin G was between .00016 to .00062 IU/ml, and an equation for the regression line was obtained: DT = 4000 × [penicillin G] + 2.8. The precision of the conductimetric technique (six replicates) was high; coefficients of variation were 2.2, 5.3, 9.5, and 8.3% for penicillin G concentrations of .00008, .00016, .00031, and .00062 IU/ml, respectively. The increased coefficients of variation at higher antibiotic concentrations might be partly due to the much smaller slopes of the conductance curves (Figure 3; curves D, E, and F). The small slopes made the determination of an accurate DT more difficult, resulting in larger errors and, hence, higher coefficients of variation. However, for all concentrations in the quantitative range (.00016 to .00062 IU/ml), the coefficients of variation were <10% and were acceptable for trace analysis.

Compared with the quantitative B. stearothermophilus disc assay (1), which uses three plates and multiple paper discs to obtain

Figure 4. Linear relationship between detection time (DT; six replicates) and penicillin G concentration in nonfat dry milk samples: A, .00008 IU/ml (DT 3.18 ± .07 h); B, .00015 IU/ml (DT 3.38 ± .18 h); C, .00031 IU/ml (DT 3.98 ± .38 h); and D, .00062 IU/ml (DT 5.33 ± .40 h).
enough data points for statistical analysis (paired t test), the quantitation of penicillin G using the conductimetric method is much easier. Because of the high sensitivity and the relatively small range useful for quantitative analysis by the conductimetric technique (Figure 4), milk samples having penicillin G concentrations >0.00062 IU/ml should be appropriately diluted for quantitative purposes.

Penicillin G in Milk Samples

After establishment of the conditions for conductance measurement, the technique was applied to test 12 market milk products, including 6 dry milk powders and 6 infant formulas. As shown in Table 1, 6 samples (3 dry milk products and 3 infant formulas) were contaminated with β-lactam antibiotics, ranging from 0.01 to 0.08 IU/g (potency equivalent to penicillin G), and were confirmed by the treatment with penicillinase. Three other samples (2 milk powders and 1 infant formula) were contaminated by inhibitors other than β-lactams, because the DT could not be restored or shortened after treatment with penicillinase. However, because of the relatively low sensitivity of the B. stearothermophilus disc assay (detection limit 0.005 IU/ml; i.e., 0.05 IU/g milk powder), only 1 sample (infant formula E) was able to produce inhibition zones by the disc assay. Obviously, the present conductimetric method can detect low concentrations of penicillin G in milk products that would be negative by the currently used assays.

CONCLUSIONS

Over the past 50 yr, a number of qualitative and quantitative methods for detecting antibiotic residues and drugs in milk and dairy products have been developed. To develop automatic, sensitive, and more rapid tests is of major interest and is the future trend of methods development. In this study, a highly sensitive conductimetric method was developed using a Malthus 2000 microbiological analyzer for the detection of penicillin G in milk products. The sensitivity of the method for penicillin G is 0.00016 IU/ml with a DT of about 3.4 h. This test is the most sensitive method ever published and may be useful to detect very low concentrations of penicillin G in milk products. Although the range that is good for quantitative analysis of penicillin G is relatively small (0.00016 to 0.00062 IU/ml), quantitation of the antibiotic in milk products with the conductance technique is easy and reproducible and avoids the labor-intensive measurement of inhibition zones produced on agar plates. In addition, automation and high sample capacity (120 or 240 samples) are other advantages of the conductance instrument. Although the application of the method in the dairy industry needs more exploration, the conductimetric method shows promise as an efficient way for analyzing milk products contaminated with antibiotics and other inhibitors.

Cogan (6) reported that a penicillin concentration of .2 IU/ml produced a 50% inhibition of cheese and yogurt starter cultures. Therefore, the currently used detection methods are generally sensitive enough for the dairy industry. However, the sensitivity of the conductimetric assay can be reduced by modifying the assay conditions (e.g., media, spore concentrations, incubation temperatures, or even test

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**TABLE 1. Detection of penicillin G in milk powders and infant formulas by conductimetric method.**

<table>
<thead>
<tr>
<th>Product</th>
<th>Detection time</th>
<th>β-Lactam antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(h)</td>
<td>(IU/g)</td>
</tr>
<tr>
<td>Milk powder</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>&gt;24</td>
<td>0.057</td>
</tr>
<tr>
<td>B</td>
<td>&gt;24</td>
<td>0.045</td>
</tr>
<tr>
<td>C</td>
<td>&gt;24</td>
<td>0.064</td>
</tr>
<tr>
<td>D</td>
<td>2.6</td>
<td>ND&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>E</td>
<td>&gt;24</td>
<td>U&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>F</td>
<td>&gt;24</td>
<td>U</td>
</tr>
<tr>
<td>Infant formula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>&gt;24</td>
<td>0.010</td>
</tr>
<tr>
<td>B</td>
<td>2.6</td>
<td>ND</td>
</tr>
<tr>
<td>C</td>
<td>2.6</td>
<td>ND</td>
</tr>
<tr>
<td>D</td>
<td>&gt;24</td>
<td>0.023</td>
</tr>
<tr>
<td>E&lt;sup&gt;3&lt;/sup&gt;</td>
<td>&gt;24</td>
<td>0.080</td>
</tr>
<tr>
<td>F</td>
<td>&gt;24</td>
<td>U</td>
</tr>
</tbody>
</table>

<sup>1</sup>Samples reconstituted 1:10 (wt/vol) in deionized water.

<sup>2</sup>Potency equivalent to penicillin G.

<sup>3</sup>No inhibitors detected.

<sup>4</sup>Containing unidentified inhibitors other than β-lactams.

<sup>5</sup>Except for infant formula E, other samples were negative by the Bacillus stearothermophilus disc assay.

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bacteria) to achieve a “reasonable” sensitivity for practical use in the dairy industry.

A plus-minus mass screen test is also possible by setting a DT threshold. Samples with DT higher than this threshold are considered to be inhibitor-positive. Because *B. stearothermophilus* is also sensitive to other antibiotic families, including tetracyclines, erythromycin, streptomycin, novobiocin, and chloramphenicol (5), the conductimetric method also may have potential to detect these residues in dairy products.

**ACKNOWLEDGMENTS**

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