ABSTRACT

Methods for antibiotic residue detection in dairy products, especially raw milk, have greatly improved as to their rapidity, accuracy and sensitivity over the past 30 years. An assay requiring overnight coagulation was available in the mid-1950’s, whereas now there is an immunologically-based test using monoclonal antibody technology requiring only 6 min. These advances have not come about without extensive research efforts. The following is an overview of the developments and their significance to the dairy industry.

Antibiotics have been used in dairy cattle management for more than three decades. They are administered to cattle by any of four general methods: (a) infusion into the udder for treatment of mastitis, (b) injection (intramuscular, intravenous or subcutaneous) for treatment of numerous diseases, (c) orally for treatment of diseases or as a dietary supplement and (d) reproductive “flush” for uterine, cervical and vaginal infections. Such uses have led to the contamination of milk and milk products with antibiotics. Use of infusible antibiotic products for treatment of mastitis in cows is a public health concern because such products are sometimes not used according to label direction. Improper milk discard times and/or multiple dosing with the same or another product can create drug residue problems (2). In addition, residues can occur after proper adherence to withdrawal times but the occurrence of residues from this cause is relatively rare (29).

The Food and Drug Administration (FDA) considers antibiotic-contaminated milk as adulterated. The FDA has attempted to reduce adulteration by limiting the quantity of antibiotics in each preparation to be used for mastitis therapy and by a requirement that a warning against the use of milk from recently treated animals be placed on the preparation (36).

The presence of antibiotics in milk in even minute quantities has created problems in the dairy industry including: (a) inadequate curdling of milk and improper ripening of cheeses during their manufacture, (b) decreased acid and flavor production during the manufacture of buttermilk and similar products, (c) diminished starter culture growth when propagated in reconstituted skim milk and (d) validity of certain quality control tests (37).

The major problem facing the dairy industry in the area of antibiotic treatment is the rapid, accurate detection of low levels of antibiotic residues in milk.

EARLY WORK

The ability of bacteria to produce acid, reduce dyes or produce a lawn of growth on agar media, has served as the basis for most tests used in the detection of antibiotics in milk. One of the earliest methods for detection of antibiotics (penicillin) in milk, based on acid production using sterile litmus, employed a culture of Streptococcus agalactiae (56). Samples were incubated at 37°C for 3 d and checked for color development. A definite pink color was considered evidence of growth, thus, the absence of penicillin.

Streptococcus lactis was used in several studies in the mid-1950’s. Lemoigne et al. (34) used four different strains of S. lactis which were each added to a portion of the milk to be tested, held overnight at 30°C, and observed for coagulation. Strains of S. lactis used were: (a) sensitive to penicillin or streptomycin, (b) resistant to penicillin, (c) resistant to streptomycin and (d) resistant to both. The presence or absence of these antibiotics was indicated by the strain or strains of S. lactis which did or did not coagulate milk. A similar method by Pien et al. (45) was based on differences in acid production when S. lactis was grown in sterile antibiotic-free milk and in antibiotic-contaminated milk. The quantity of antibiotic in each milk was determined by the use of S. lactis strains resistant to different concentrations of the antibiotic.
Dopter (13) suggested the use of strains of *S. lactis* resistant to various concentrations of different antibiotics as a method for quantitative detection in milk of penicillin, streptomycin or chlorotetracycline by coagulation after 12 h of incubation. Bertridge (7) developed a rapid method (15 min) for detection of selected antibiotics in milk based on the measurement of very small changes in pH brought about by growth of *S. lactis*, but unfortunately penicillin could not be detected by this method.

From earlier work, Bertridge (5,6) described a method in which a culture of *Streptococcus thermophilus* plus bromcresol purple was added in equal volume to the milk sample. The mixture was incubated at 45°C and examined at 30-min intervals for changes in color. A color change after 30 min indicated 0.015 to 0.06 unit of penicillin present per ml, whereas a color change at 1 h or later indicated less than 0.015 unit per ml. However, maintaining the culture in log-phase proved to be a problem. Collins (12) tested this method with diluted culture and detected the presence, per ml, of 0.02 unit penicillin, 0.5 µg chlorotetracycline, 6.5 µg streptomycin, 0.7 µg oxytetracycline or 0.9 µg tetracycline.

A 2.5-h test for antibiotics was described by Ullberg (54) which employed a strain of *Lactobacillus bulgaricus* sensitive to 0.01 unit of penicillin per ml. The organism normally coagulated milk in the allotted time when incubated at 45°C, but failed to do so when penicillin was present in the sample.

Many authors have recommended starter cultures for detecting antibiotics in milk. Ruehe (47) suggested that 10-ml samples of milk, after heating to 79.4°C for 5 min and cooling to 22.2°C, be inoculated with 1 ml of a good starter culture. If penicillin were absent, a satisfactory coagulum forms in <10 h. Krienke (32,33) and Hansen et al. (20) used buttermilk cultures to detect antibiotics. Yogurt cultures were suggested for use in detecting antibiotics in milk by Fleischmann (16) and Adamse (1). It was claimed the yogurt culture was 10 times more sensitive to penicillin than other starters, and could detect 0.005 unit per ml.

Schipper and Peterson (49) used methylene blue and a culture of *Bacillus cereus* var. *mycoides* to detect chlorotetracycline in milk at 0.031 µg per ml. The method was modified by Schipper and Peterson (50) using *Bacillus mesentericus* for detecting chlorotetracycline and oxytetracycline in milk. Galesloot (18) was able to detect penicillin in milk at concentrations as low as 0.01 unit per ml using methylene blue solution and *S. thermophilus*, whereas Bertelsen and Mattson (8) were able to detect 0.05 unit of penicillin by use of methylene blue and a yogurt culture.

A method to detect penicillin in milk with resazurin and *S. thermophilus* was reported by Hietarananta and Timroth (22), but Slatter (52) found the resazurin method did not distinguish between different levels of penicillin in samples of pasteurized milk.

The triphenyltetrazolium method for detecting inhibitory substances in milk was described by Neal and Calbert (40) and Neal (39). The test is based on conversion of 2,3,5-triphenyltetrazolium chloride (TTC) to formazan by actively growing bacterial cells. This conversion is accompanied by a color change from the yellowish leucoform to red. A *S. thermophilus* culture was used, and incubated at 37°C for 2.5 h. The test detected the presence of 0.04 unit of penicillin in milk, but sanitizing agents were found to interfere with the test. Dragon (14) suggested a modification to the TTC test which enabled one to determine, by gas formation from glucose and yeast cultures, whether the inhibition resulted from antibiotics or sanitizers. The TTC test was used for detection of antibiotics in dried milk by Kotter and Muspack (31) with a yogurt culture being substituted for *S. thermophilus*. Parks and Doan (44) compared sensitivities of the TTC test and disc assay for various antibiotics. The TTC test was about equal in sensitivity to the disc assay method for detection of penicillin and chlorotetracycline, but was less sensitive to streptomycin and totally unsatisfactory for detection on neomycin. A more rapid TTC test was introduced by Igarashi et al. (23) which employed *Bacillus stearothermophilus* and an incubation temperature of 62°C. The test, completed after about 45 min of incubation, attempted to detect the presence of 0.005 unit of penicillin per ml of milk, but later results showed that neither the sensitivity nor the incubation time claimed could be achieved (24).

**CYLINDER-PLATE METHOD**

Foster and Woodruff (17) described a method in which 15,000 spores of *Bacillus subtilis* were added per ml of an agar medium after which 13 ml of the seeded agar were added to a petri dish. Small, sterile glass cups (cut from tubing) were warmed and set lightly on the agar. These cups were filled with the sample or a standard solution. Plates were then incubated at 30°C for 12 to 16 h. The area around the cup in which microbial growth did not occur (zone of inhibition) was measured, and that of the sample compared with that of a standard solution. This assay became known as the cylinder-plate method.

Schmidt and Moyer (51) suggested *Staphylococcus aureus* for assaying penicillin by means of the cylinder-plate method. They reported the size of the zone decreased as the depth of the agar increased. This method was modified by Beadle et al. (4) who suggested use of 104 x 292-mm rectangular glass plates instead of petri dishes. Factors which affected the cylinder-plate test when applied to milk were studied by Meewes and Milosevic (38). They found zones of inhibition produced by penicillin increased with incubation time and decreased with the depth of agar. Raw milk that contained no penicillin sometimes inhibited *S. aureus*. Other organisms have been suggested for use in cylinder-plate procedures. Among these is *Sarcina lutea* for detection of penicillin, which was recognized as the organism used in the cylinder-plate method that became the “official” procedure of FDA in 1955 (28).
One of the earliest disc-plate methods introduced was that of Welsh et al. (57) which employed 3 ml of agar seeded with spores of *Bacillus subtilis*, 7-mm filter paper discs to which 0.031 ml of test liquid was added, with an incubation temperature of 39°C for 4 h. Drury (15) described a procedure which was commonly used for many years. This method employed the following regime: (a) whey agar seeded with *B. subtilis* spores at a final concentration of 250,000 spores per ml of agar, (b) 10 ml of seeded agar, (c) 7-mm paper discs soaked in milk (ca. 20 μl milk) and placed on surface of seeded agar, and (d) incubation at 37°C for 4 h. This procedure was able to detect 0.05 unit of penicillin per ml of milk. Gogas and Bicknell (20) described a disc-plate method to detect penicillin in milk which provided results in ~2 h using *B. subtilis* in Penassay agar (Difco) using 13-mm discs incubated at 37°C. Johns and Berzins (26) studied the method just described and found zones could only be detected in 2 h if fresh plates were used. When previously incubated plates were refrigerated overnight, zones could first be detected after 5 h of incubation.

Wolin and Kosikowski (58,59) studied the effect of natural inhibitory substances in milk on disc assay methods. Dried milk (lyophilized) discs prepared from milks containing the inhibitory substances produced larger zones than milk-soaked paper discs. Sensitivity of the disc-plate method as affected by discs was studied by Suno et al. (53). He found the test more sensitive when 13-mm discs were used instead of 7-mm discs. Arret and Kirshbaum (3) reported a disc assay method that could purportedly detect 0.05 unit of penicillin per ml of milk in 2.5 h using *B. subtilis* with plates incubated at 37°C. Johns (25) studied this method and concluded it was less reliable and less sensitive than existing disc assay methods.

In 1960, Igarashi (23) suggested a rapid disc-plate method which employed trypticase-yeast extract-glucose agar, *B. stearothermophilus*, 13-mm discs and incubation at 65°C for 1.5 h. The optimum incubation period was found to be 2.5 h and the method could detect 0.005 unit of penicillin per ml of milk. Igarashi (24) reported that he was able to detect 0.002 unit of penicillin per ml of milk by incorporating 2,3,5-triphenyltetrazolium chloride in the agar.

The majority of original research in this area was done during a period from about 1945 to 1961. Studies undertaken after this period were devoted primarily to perfecting these existing methods by testing their accuracy and precision, and incorporating factors into the assay to improve rapidity and ease of performance. Palmer (43) studied the effect of sanitizers on antibiotic disc assay testing of milk. Results indicated no interference when residual sanitizers were present even at much higher concentrations than would be expected under good operational practices.

Medical concern about the potential health hazard of natural foods contaminated with even traces of penicillin led to a reevaluation in 1967 of the 0.05 unit of penicillin per ml concept for penicillin residues in milk (30). As a result of this, three sensitive assay procedures were introduced in the 1970's.

**CHARM TEST**

The Charm test, introduced in 1978 by Penicillin Assay, Inc., is a product of modern biochemical technology. It is a radioimmunoassay method that is able to detect 0.005 unit of penicillin per ml of fluid milk in just 25 min. Low level radioactive 14C and a binder were added to the milk sample. The presence of penicillin interferes with the association of the 14C to the binder sites. Since the penicillin interferes with the binding process, the lower the concentration of penicillin, the higher the digital readout from the Geiger counter (11). Current industry estimates indicate that more than 70% of all U.S. raw milk is screened using this test (48).

**DELVOTEST-P**

The Delvotest-P was introduced in 1975 by Gist-Brocades Laboratories in the Netherlands. It is an agar diffusion test. Tablets containing nutrients and bromcresol purple are added to ampules containing plain agar with spores of *Bacillus stearothermophilus* var. *calidolactis* (10). After adding 0.1 ml of milk sample, the ampule is incubated for 2.5 h at 63 to 66°C. In the absence of antimicrobial substances, the whole of the solid medium turns yellow (negative result), whereas it remains purple in the presence of sufficiently high concentrations of antibiotics (positive result). At intermediate concentrations of antibiotic, the solid medium turns partly yellow (doubtful result). The test method is highly sensitive for penicillin. Concentrations of 0.002 unit of penicillin per ml or lower give negative results in all samples, and 0.003, 0.004, and 0.005 unit per ml predominately negative, doubtful, and positive results, respectively. These results are obtained in bulk milk samples, and in individual milk samples. The method is also sufficiently sensitive to most of the other antibiotics usually administered to lactating cows (55). Packard (42) and Johnson et al. (27) compared the Delvotest-P with the S. lutea cylinder-plate method and the *B. subtilis* disc assay method. A greater number of positive detections of penicillin in milk were found by the Delvotest-P than the other two procedures.

**BACILLUS STEAROTHERMOPHILUS DISC ASSAY**

A sensitive disc assay using *B. stearothermophilus* var. *calidolactis* was introduced by Kaufman (30) in 1977. It employs antibiotic medium #4 (Difco) seeded with *B. stearothermophilus* spores. The seeded agar (6 ml) is put into each 15 x 100-mm plastic petri dish. Plates must be used within 7 d of the date prepared. Paper discs (12.7 mm) are soaked with the milk sample by capillary action and placed on the agar. The plate is incubated for 2.5 h at 64°C. An alternate method is for incubation to proceed
for 4 h at 55°C. After incubation is completed, zones of inhibition are then measured in mm. A positive result, or actionable level, is designated by a zone size of 16 mm (2). This assay detects as little as 0.005 unit of penicillin per ml of milk. From the data, it appears that this method is considerably more sensitive for milk than either the disc assay using B. subtilis or the cylinder-plate method, is much simpler than the cylinder assay, and is readily adapted to the dairy laboratory. This assay, approved by the National Conference on Interstate Milk Shipments (NCIMS) in May, 1981, and then accepted by FDA, was made official by the NCIMS executive board, effective January 1, 1982 (2).

Antibiotic medium #4 (Difco) incubated at 64°C is preferred for the B. stearothermophilus disc assay. This medium yielded a significantly (P<0.05) higher percentage of positive values than PMI agar (46). Ginn et al. (19) described a method for making quantitative estimates of β-lactam residues relative to a fixed reference standard using the B. stearothermophilus disc assay. Quantitative estimates above or below the reference concentration of antibiotic are computed through a paired-t statistical analysis.

**COMPARATIVE STUDIES**

As illustrated in Table 1, minimum concentrations of penicillin-G detected by some of the more commonly used procedures varies. MacCaulay (35) compared the Delvotest-P assay and the B. stearothermophilus disc assay and obtained results showing approximately equal sensitivity, but the Delvotest-P produced 11% false-positives (Table 2).

Bishop (9) found the B. stearothermophilus disc assay and the Delvotest-P had similar accuracy and precision. The only difference was the possibility of a doubtful result with the Delvotest-P which is not possible with the B. stearothermophilus disc assay (Table 3). Also accomplished in this study was the quantitative of some of the more commonly used antibiotics for treatment of mastitis, i.e., penicillin, cephalosporin and oxytetracycline. This was derived with the B. stearothermophilus disc assay be establishing a relationship between the log10 value of the antibiotic concentration and the respective zone of inhibition produced (mm). These relationships include:

\[ \log_{10} \text{penicillin conc.} = (\text{zone} - 35.587)/8.628 \]
\[ \log_{10} \text{cephalosporin conc.} = (\text{zone} - 31.823)/7.674 \]
\[ \log_{10} \text{oxytetracycline conc.} = (\text{zone} - 22.282)/7.638. \]

**MOST RECENT METHODS**

Angenics, Inc., has developed a 6-min test for antibiotic residues in milk. The test is immunologically-based using current monoclonal antibody technology. The manufacturer anticipates providing detection methodology to all sectors of the dairy industry by supplying farm, bulk hauler and receiving station test kits. The receiving station kit contains a sampling syringe with a predetermined amount of buffer. The syringe is used to collect the milk sample which is then mixed with two reagents and placed on a glass slide. This slide is inserted into the reaction-monitoring device and antibiotic contamination is evaluated. Fine tuning by the manufacturer is said to produce results equivalent to those obtained by the B. stearothermophilus disc assay. Bulk hauler and farm tests are similar to the receiving station kit except that reagents are pre-dried on the glass slide. Pre-drying the reagents results in decreased sensitivity for these two test. Shelf-life of these kits is expected to range from 6 to 12 months.

The Penzym test is an enzymatic colorimetric screening method for the rapid detection of β-lactam antibiotics in milk. The total test time is 20 min, and is currently being used to screen incoming raw milk in at least one Canadian dairy (48).

**TABLE 1. Minimum concentrations of penicillin-G detected by various assays.**

<table>
<thead>
<tr>
<th>Methods</th>
<th>Penicillin-G (Unit/ml)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sarcina lutea</em></td>
<td>0.020</td>
<td>28</td>
</tr>
<tr>
<td>cylinder-plate</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em> disc</td>
<td>0.050</td>
<td>15</td>
</tr>
<tr>
<td>assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Charm test</td>
<td>0.005</td>
<td>11</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em> disc assay</td>
<td>0.005</td>
<td>30</td>
</tr>
<tr>
<td>Delvotest-P</td>
<td>0.005</td>
<td>55</td>
</tr>
</tbody>
</table>

**TABLE 2. Percentage of positive detection of penicillin using the Delvotest-P and B. stearothermophilus disc assay (35).**

<table>
<thead>
<tr>
<th>Penicillin conc. (Unit/ml)</th>
<th>Delvotest-P Percentage of positive detections</th>
<th>B. stearothermophilus disc assay Percentage of positive detections</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.050</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.0250</td>
<td>100</td>
<td>89</td>
</tr>
<tr>
<td>0.0100</td>
<td>89</td>
<td>89</td>
</tr>
<tr>
<td>0.0050</td>
<td>67</td>
<td>89</td>
</tr>
<tr>
<td>0.0025</td>
<td>78</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>11</td>
<td>0</td>
</tr>
</tbody>
</table>

**TABLE 3. Comparison of the Delvotest-P and B. stearothermophilus disc assay for known concentrations of penicillin in milk and unknown individual cow samples of milk.**

<table>
<thead>
<tr>
<th>Penicillin conc. (Unit/ml)</th>
<th>Delvotest-P Percentage of positive detections</th>
<th>B. stearothermophilus disc assay Percentage of positive detections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk samples (N = 308)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)a</td>
<td>91</td>
<td>97</td>
</tr>
<tr>
<td>(+/-)</td>
<td>11</td>
<td>N/Aa</td>
</tr>
<tr>
<td>(-)</td>
<td>206</td>
<td>211</td>
</tr>
</tbody>
</table>

*a(+), positive detection of antibiotic residues. (+/-), doubtful detection of antibiotic residues. (-), negative detection of antibiotic residues. 
N/A, not applicable.
CONCLUSIONS

Methodology is now available for the rapid, sensitive and relatively simple detection of antibiotic residues in milk. There are also procedures which will now allow the estimation of the concentrations of antibiotic involved. Other than improving the assays presently being used, the only remaining area to be resolved is the identification of the specific antibiotics detected by the assays. If scientific progress in the area of antibiotic detection continues at its present rate, this information will be obtained in the near future.

ACKNOWLEDGMENTS

The authors are not advocating the use of any of the previously mentioned methods for antibiotic residue detection of antibiotics.

REFERENCES

52. Slatter, W. L. 1954. Comparison of the resazurin and the acidity tests for detecting the presence of antibiotics in pasteurized milk. Milk Dealer 43:60.