

## A Practical Sensitive Test to Detect Penicillin in Milk

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### ABSTRACT

The disc assay of the International Dairy Federation for determination of penicillin in milk has been modified in the following steps: preparation of seed broth, seeded plates, and standard solutions. The modified assay is sensitive and relatively rapid, requiring a 4-h incubation at 55 C. Approximately 0.004 unit of penicillin/ml of milk can be detected on plates stored for 7 days; with freshly prepared plates and standard solutions, as little as 0.002 unit of penicillin/ml can be detected.

The sensitivity of the American Public Health Association disc assay (1) for penicillin in milk is about 0.05 unit/ml, using *Bacillus subtilis* in the 14 to 24-h assay at 32 C. A more sensitive test which requires less time and which is practical and economical is needed. The International Dairy Federation (IDF) at its meeting in Australia in October, 1970 approved publication of a disc assay procedure for detection of penicillin in milk; this procedure (3), which specifies *Bacillus stearothermophilus* var. *calidolactis* as the test organism, is more sensitive (< 0.0025 unit/ml) and requires less time than the *B. subtilis* assay presently undertaken in most dairy laboratories. Assay methods specified by the Association of Official Analytical Chemists include both a disc and a cylinder technique (2). The disc assay is sensitive to approximately 0.05 unit of penicillin/ml while the cylinder assay, which is more complex to use, is suitable for concentrations of less than 0.025 unit/ml.

We undertook some studies of the IDF procedure. Slight modifications were made to serve the practical needs of the dairy laboratory, i.e., in preparation of seed broth, standard solutions, and seeded plates. The test as described is sensitive, fits nicely into the 5-day work week, and provides assay plates for a 7-day period.

### MATERIALS AND METHODS

The test organism was *B. stearothermophilus* var. *calidolactis*. The stock culture was maintained in a screw-cap tube on a medium (slant) consisting of 2 g of yeast extract, 5 g of peptone, 1 g of meat extract, 5 g of sodium chloride, 15 g of agar, and 1000 ml of distilled water; the final pH was 7.4 ± 0.1. The stock culture was prepared by streaking

and incubating the above medium for 48 h at 55 C; the culture was stored at 4 C. To start the test using the agar slant stock culture, transfer a loopful of slant culture to 50 ml of seed broth; incubate for 18 h and refrigerate for 5 days. This 5-day old culture is used to prepare seed broth as described below. The seed broth used to culture the organism contained 10 g of yeast extract, 20 g of tryptone, 0.5 g of glucose, and 1000 ml of distilled water; the final pH was 8.0 ± 0.1. The following regimen was followed to prepare the seed broth culture. Inoculate 50 ml of seed broth in a milk dilution bottle with 0.1 ml of a 5-day-old refrigerated broth culture and incubate for 18 h; transfer 1 ml of this culture to a second bottle containing 50 ml of seed broth and incubate for 6 h; transfer 1 ml of this culture to a third bottle with 50 ml of seed broth and incubate for 18 h. Prepare seeded plates from this culture. Store the 49 ml of seed broth which remain from the last transfer above at 4 C for 5 days; use this 5-day-old culture as the source of the inoculum for the first bottle. With the above procedure, the stock cell suspension counts of the seed broth ranged from 150,000 to 1,000,000/ml. Using our technique, we were unable to achieve the counts obtained by the IDF assay (50 to 100 million), but we were able to obtain clear zones of inhibition on the plates.

Plate Count Agar (PCA) adjusted to pH 8.0 ± 0.1 was used for preparing the seeded plate. Petri plates with bottoms having an inside diameter of 85 mm were used. To make a seeded plate, 12 ml of seed broth was added to 60 ml of PCA agar at 55 C; 7 ml of this mixture was used. Prepared plates were used immediately or stored for up to 8 days in a plastic sleeve at 5 C. No attempt was made to make the sleeve anaerobic.

The standard stock solution of potassium penicillin G solution was prepared to contain 10,000 units/ml in pH 6.5 buffer; the buffer was made by dissolving 8 g of monobasic potassium phosphate and 2 g of dibasic potassium phosphate in 1000 ml of distilled water, and adjusting the pH to 6.5. The stock solution was used immediately or stored at 5 C for not more than 24 h. On the day of use, 1 ml of the stock solution was diluted to 100 ml with pH 6.5 buffer; 1 ml of this solution was further diluted to 100 ml with buffer. Additional dilutions were made with sterilized (121 C for 10 min) pasteurized, antibiotic-free homogenized whole milk as follows: 1 ml of the second dilution was made up to 100 ml with milk; then 20-, 10-, and 5-ml volumes were each diluted to 50 ml with milk to obtain concentrations of 0.004, 0.002, and 0.001 unit of penicillin/ml, respectively.

For the assay, blank discs, ½ inch (1.27 cm) in diameter, were touched to the surface of the sample to allow the disc to soak up the sample. The edge of the disc was then touched three times on the underside of the petri dish lid; this provided a sterile surface and the milk spots could be easily removed with tissue before the top was replaced on the plate. The discs were placed on the surface of the agar as described in the *B. subtilis* assay (1). All plates were inverted and incubated at 55 C for 3.5-4 h. Sterilized, pasteurized, penicillin-free homogenized whole milk was used as the negative control.

## RESULTS AND DISCUSSION

Zone diameters were measured to the nearest millimeter. Averages of duplicate discs on triplicate plates are shown in Table 1. In only rare instances did the zone diameters vary by 1 mm.

The initial number of cells obtained on culturing by the procedure described is given in Table 1 for each trial. No explanation can be given for the range of cell numbers obtained, and no effort was made to study this aspect. The range in cell numbers actually provided the opportunity, without resorting to dilution, to determine a lower and an upper limit within which the assay could be undertaken on a practical basis. This 10-fold difference is of the same magnitude as that recommended for the *B. subtilis* disc assay (1).

The data show that the modified IDF assay can detect 0.004 unit of penicillin/ml in milk, using plates which have been stored for 8 days. The number of cells employed (150,000-1,000,000) did not influence the zone diameter when the diameter was measured to the nearest 1 mm; no effort was made to be more precise, as this represents a practical objective for most dairy laboratories.

A level of 0.002 unit/ml can be detected when fresh plates and fresh standard solutions are employed. At the 0.002 unit/ml level, clearly defined zones were observed for 66% of the assays with plates stored for 24 h and for 25% of the assays with plates stored for 7-8 days.

Clear zones of inhibition were readily seen at a penicillin concentration of 0.004 unit/ml in 3.5 h, but a 4-h incubation was needed to detect penicillin at 0.002 unit/ml. If zones are to be measured, a 4-h incubation

period is mandatory to permit formation of a zone with a distinct edge.

All incubations were done at 55 C. Use of 55-C incubation is advantageous in that very few bacterial contaminants commonly found in a dairy laboratory will grow at this temperature. If, therefore, a poor technique is used in making culture transfers to seed broth, it is not likely that the contaminant(s) could grow and confuse interpretation of the test.

On the basis of these preliminary data it is apparent that the modified IDF test is (a) considerably more sensitive than either the official disc or the cylinder assay method for milk, (b) much simpler than the cylinder assay, and (c) readily adapted to the dairy laboratory, as it utilizes the basic disc assay technique which is familiar to technicians presently engaged in antibiotic assays. It is hoped that interested groups will be encouraged to investigate this procedure more fully to determine its suitability for incorporation in the next edition of *Standard Methods for the Examination of Dairy Products*.

## ACKNOWLEDGMENT

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## REFERENCES

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TABLE 1. Detection of known levels of penicillin added to milk

Penicillin concentration (unit/ml)	Zone diameters (mm) obtained at cell concentrations indicated															
	150,000/ml				290,000/ml				610,000/ml				1,000,000/ml			
	1 (1) <sup>a</sup>	1 (0)	7 (1)	0 (0)	1 (1)	8 (1)	1 (1)	1 (0)	7 (1)	0 (0)	1 (1)	8 (1)				
0.004	16 <sup>b</sup>	16	16	16	16	15	15	15	15	17	16	16				
0.002	14	15	0	15	15	0	0	14	0	15	0	15				
0.001	0	0	0	0	0	0	0	0	0	0	0	0				
Negative	0	0	0	0	0	0	0	0	0	0	0	0				

<sup>a</sup>The first figure indicates the age of the plate in days at the time of assay; the figure in parentheses indicates the age of the standard solution.

<sup>b</sup>Zone diameters are the average of two discs on each of three plates.