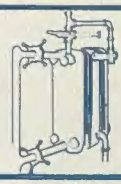
AGRICULTURAL EXTENSION SERVICE · UNIVERSITY OF MINNESOTA

MINNESOTA DAIRY PRODUCTS PROCESSOR



Editor: V.S. Packard
Extension Specialist, Dairy Products

October 1, 1981 No. 82

TESTING FOR ANTIBOITICS

Over the years, tests for antibiotics in milk and dairy products have become increasingly sensitive. In fact, we have reached a point at which a "zero tolerance" -- no detectable level -- seems unreasonably strict and unwarranted. But if a tolerance were established, if some very small level of antibiotic residue in milk were allowed, it would be necessary for control purposes to have a test procedure able to determine whether or not that level had been breached. Ideally, such a procedure would be relatively simple and quick to run.

Working on the disc assay principle of determining presence of antibiotics, Roy Ginn, Dairy Quality Control Institue, Inc.; Ronald Case, Kraft, Inc.; Dr. Sita Tatini, and I, Department of Food Science and Nutrition, University of Minnesota, developed an approach that seems to have potential in this regard. I would like to devote this issue of MDPP to a brief coverage of this technique.

THE APPROACH

The test follows closely the disc assay method utilizing Bacillus stearothermophilus as the test organism. The qualitative method, of which a commercial test is available, is modified to provide for a kind of quantitative estimate. That is, the test gives evidence of whether an unknown level of antibiotic is above or below a reference level. It does not estimate how much, in absolute terms, is present. Results can be analyzed statistically to permit a high degree of confidence -- 95 percent or higher -- in their validity. Thus the procedure will find the most appropriate application when and if a tolerance is established. Incidentally, it is valid only for penicillin (beta lactam) residues.

REFERENCE STANDARD NEEDED

Given a tolerance level of antibiotic, a reference standard made up to that precise level becomes necessary and hopefully would one day be available commercially. At present, standards would have to be prepared using pure, crystalline pencillin G sodium (or potassium). Appropriate stock is available from USP Reference Standards, 12601 Twinbrook Parkway, Rockville, MD 20852. For test purposes, crystalline penicillin is dissolved (to some pre-determined concentration) in buffered (pH 6.0) (phosphate buffer), distilled water. This stock solution is then used to prepare a reference concentration in milk.

RUNNING THE TEST

In brief, the procedure goes as follows:

1. Heat both unknown and reference milk samples to 82°C for precisely two minutes.
2. Ultimately, place six filter discs around the periphery of a seeded (B. stearothermophilus) culture plate. Use only flat petri dishes. Carry out the work only on an absolutely flat surface.
3. As filter discs are applied, touch them gently to the surface of the agar with the tips of the forceps.
4. Place a penase disc in the center of the plate.
5. Within 45 to 60 seconds after any one filter disc is placed on the seeded agar surface, add either reference or unknown sample.

6. Starting in the 12 o'clock position, add reference milk sample to the disc and alternate the reference sample with the unknown samples on the remaining discs. In other words, three identical reference samples are alternated with three identical unknown samples on the same plate.

Three such plates are ultimately prepared for each unknown sample.

The amount of sample is fixed at 90 microliters (µl). This amount is added by means of a 90 µl pipetter using disposable tips, one fresh tip for each of the three reference standards, and one for the three replicate unknown samples.

7. Invert plates and incubate at $64^{\circ} \pm 2^{\circ}$ C for two hours and 45 minutes.

8. Remove plates in sets as incubated and measure zone diameter of both reference and unknown samples to the nearest 0.1 mm. using vernier calipers. Use penase disc simply as control.

WHAT YOU HAVE FOR DATA

You eventually end up with nine measurements of zone diameters each for the reference and unknown samples. If the antibiotic concentration in the unknown is higher than the reference, you would expect that fact to be reflected in larger zone sizes on the average. Or, conversely, a smaller concentration would yield smaller zones than the reference samples.

It is here, then, that statistics must be applied to confirm with some degree of confidence whether or not the unknown zone diameters are in fact larger or smaller than those of the reference standard. The first step in this process is to determine the difference in zone size between each reference standard and the unknown immediately adjacent to it, clockwise around the plate. Thus each plate yields three differences; three plates provide nine differences.

STATISTICAL ANALYSIS

A shortened version of the paired - t formula is used for analyzing data. Assuming that nine differences are determined, the formula becomes:

$$t = \frac{2.828 \times D_1}{\sqrt{9 \times D_2 - (D_1)^2}}$$

In the above formula, D_1 is the sum of the nine differences obtained over the three plates. D_2 is the sum of the squared differences of the nine pieces of data. The constant, 2.828, remains valid only for a paired - t analysis involving nine pairings.

THE MEANING OF "t"

Having derived a value for "t," it is necessary only to compare that figure with a known value for "t" at some given level of confidence. At 95 percent confidence, the known value in the above analysis is 1.860.

If the derived "t" is larger than 1.860, you are assured of the validity of your findings at 95 percent confidence. Assume, for example, that the unknown zone sizes were found to be generally larger than the reference zones. A derived t-value above 1.860 gives you 95 percent confidence that the concentration is in fact higher than the reference standard. The reverse of that situation can likewise be verified, i.e., a lower concentration detected.

Using a reference concentration of 0.015 units of penicillin per milliliter, our results suggest a potential to consistently detect, at 95 percent confidence, concentrations of penicillin 0.003 units/ml different from that standard. That is, you could readily detect a concentration of 0.018 units/ml as actually being above the standard. You could as easily validate as lower than the standard a concentration of 0.012 units/ml. The test, therefore, has rather precise powers of discrimination. That is a distinct advantage, especially in the event that a tolerance is established.

SLIDE SET, CONSULTATION

We are in the process of preparing a slide set(s) that describes in detail the step-by-step procedure for running this test. We plan to make the set (with script or tape) available to interested persons or laboratories. As time and finances permit, I would be happy to visit your lab and discuss details.

Vernal S. Packard

Vernal S. Packard, Jr.
Extension Specialist, Dairy Products.