Detection of Sulfa Drugs and Antibiotics in Milk

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A disc assay method for testing sulfa drugs and antibiotics in milk was developed wherein Bacillus megaterium ATCC 9855 was used as the test organism and Mueller-Hinton agar was used as the test substrate. Incubation was at 37 C for 4 to 5 hr. The test procedure is an improvement over the Bacillus subtilis-antibiotic Medium No. 1 method, as described in Standard Methods for the Examination of Dairy Products, in that it is sensitive to eight sulfa drugs and to bacitracin without a significant change in sensitivity to eight other antibiotics commonly used for mastitis therapy.

The Grade "A" Pasteurized Milk Ordinance of the Food and Drug Administration (4) requires that producers' milk or commingled milk be tested for antibiotics at least four times during any consecutive 6-month period. Although sulfa drugs are commonly used along with antibiotics to treat bovine mastitis, the test procedure used (1) is generally insensitive to sulfa drugs. Chemical methods are available for detecting sulfa drugs in milk; however, the analytical procedures involved are far more complex than the antibiotic test now in use for routine regulatory testing (3, 5).

We believed it would be desirable to develop a procedure for routine regulatory testing that would be useful in detecting sulfa drugs as well as the antibiotics commonly used to treat mastitis, provided that the test developed was not more complex than the Bacillus subtilis method described in Standard Methods for the Examination of Dairy Products (1). This paper reports the development of a testing procedure that meets this requirement.

MATERIALS AND METHODS

Preparation of assay plates. Three sporeforming and two nonsporeforming organisms were studied as candidate test organisms for sensitivity testing. These were B. subtilis ATCC 6633, B. cereus ATCC 11778, B. megaterium ATCC 9855, Sarcina lutea ATCC 9341, and Escherichia coli ATCC 11229. B. subtilis spores were obtained from Difco Laboratories, Inc. Spore suspensions of B. cereus and B. megaterium were prepared by growing the cells in AK Sporulation Medium No. 2 (BBL) in 6-oz prescription bottles incubated for 48 hr at 35 C. Suspensions of S. lutea and E. coli were prepared daily from slants of Mueller-Hinton agar that had been incubated for 18 to 20 hr at 32 C. Three successive transfers were made before the cells were harvested and used. Growth from sporeforming or vegetative cell cultures was washed from the agar with phosphate-buffered distilled water and centrifuged at a relative centrifugal force of 5,000 for 15 min at 3 C, and the centrifugation-washing process was repeated three times. Spore crops were stored in buffered dilution water at 4 C until used. All inocula (vegetative cells or spores) were adjusted in optical density to give a final concentration of about 5 × 10⁸ per ml of agar. The inoculum was added to the agar at 50 C, the inoculated agar was mixed gently by swirling to avoid air-bubble formation, 4 ml of agar was pipetted into 90-mm inside diameter plastic petri dishes, and the agar was distributed over the dish by swirling the dish. Fresh agar was prepared for each day of testing, and the spores, when used, were not heat-shocked.

Preparation and testing of milk samples. Farm-bulk-tank or dairy-storage-tank raw milk was used for this study. Discs containing a sulfa derivative or an antibiotic were prepared by weighing the inhibitor under test and suspending it in distilled water. From this, an appropriate amount was added to milk, and two-fold serial dilutions were made of the inhibitor in milk. One-tenth milliliter of milk containing the inhibitor was added to 0.5-inch (12.7-mm) blank discs (Carl Schleicher and Schuell Co.), and the discs were placed on the inoculated solidified agar for inhibitory testing. Temperatures of 25, 32, 35, and 37 C were used for various incubation times. Zone measurements were made with a vernier caliper with the plates illuminated from the back by fluorescent light. Zones, 15 mm in diameter or larger, were recorded as positive (disc diameter is 12.7 mm). For routine testing, any zone should be recorded as positive providing the proper controls are run. The 15-mm criterion was used in this study because it gave a zone that could not be mistaken even by a relatively untrained analyst. All minimum sensitivities reported were obtained in at least two trials.

RESULTS AND DISCUSSION

Eight sulfa drugs and nine antibiotics were selected for study, and the inhibitor testing procedure listed in Standard Methods for the Ex-
amination of Dairy Products was used to establish the sensitivity of the procedure now in general use for regulatory testing to the inhibitors selected (Table 1). We believe that the general insensitivity of the standard method to sulfa drugs was the result of the medium (Antibiotic Medium No. 1) containing p-aminobenzoic acid or folic acid or both. Since *B. subtilis* produces bacitracin, it is insensitive to it.

Mueller-Hinton agar has been widely used for the detection of sulfa drugs, and this medium was selected to test the sensitivity of five species of bacteria to the 17 inhibitors under study (Table 2). In all cases, incubation was at 37°C for a previously determined incubation time that gave optimal zones with the test organism. *S. lutea* and *B. megaterium* were more sensitive to the test inhibitors than were the other three organisms tested. *B. megaterium* was selected for further study inasmuch as a sporeformer is preferable to a nonsporeformer for routine testing because it can be purchased in ready-to-use form and stored until used. This would make the test easier to standardize between laboratories. Heat shocking the spores of *B. cereus*, *B. subtilis*, and *B. megaterium* did not enhance sensitivity or significantly reduce assay time.

Since 37°C incubation is not commonly used in milk testing laboratories, incubation temperatures of 35, 32, and 25 were studied to determine whether they might be as useful as 37°C

### Table 1. Sensitivity of the standard *B. subtilis* assay procedure for inhibitors to sulfa drugs and antibiotics

| Inhibitor         | Minimum detectable concn (μg/disc) | Inhibitor         | Minimum detectable concn (μg/disc) |
|-------------------|-----------------------------------|-------------------|-----------------------------------|
| Sulfamethazine    | 5.0                               | Tetracycline      | 0.04                             |
| Sulfamethizole    | 5.0                               | Penicillin G      | 0.005                            |
| Sulfamerazine     | 2.5                               | Chlortetracycline | 0.08                             |
| Sulfathiazole     | 1.3                               | Neomycin          | 2.5                              |
| Sulfadiazine      | 1.3                               | Oxytetracycline   | 0.08                             |
| Sulfisomidine     | 5.0                               | Nitrofurantoin    | 5.0                              |
| Sulfisoxazole     | 5.0                               | Erythromycin      | 0.02                             |
| Sulfanilamide     | 10.0                              | Novobiocin        | 0.16                             |
|                   |                                   | Bacitracin        | >12.50*                         |

* Units per disc.

### Table 2. Sensitivity of five test organisms to sulfa drugs and antibiotics when incubated at 37°C in Mueller-Hinton agar

| Inhibitor         | Test organism                  |
|-------------------|--------------------------------|
|                   | *Escherichia coli* (6)*a        | *Sarcina lutea* (10)*a | *Bacillus subtilis* (6)*a | *Bacillus cereus* (6)*a | *Bacillus megaterium* (5)*a |
| Sulfamethazine    | 0.08                           | 0.08                      | 0.62 | 0.62 | 0.08 |
| Sulfamethizole    | 0.16                           | 0.08                      | 0.31 | 0.31 | 0.16 |
| Sulfamerazine     | 0.04                           | 0.08                      | 0.31 | 0.31 | 0.04 |
| Sulfathiazole     | 0.08                           | 0.04                      | 0.62 | 0.62 | 0.04 |
| Sulfadiazine      | 0.04                           | 0.04                      | 0.16 | 0.31 | 0.08 |
| Sulfisomidine     | 0.08                           | 0.08                      | 0.62 | 0.62 | 0.04 |
| Sulfisoxazole     | 0.08                           | 0.04                      | 0.62 | 0.16 | 0.04 |
| Sulfanilamide     | 5.00                           | 0.16                      | 0.16 | 5.00 | 0.08 |
| Tetracycline      | 0.16                           | 0.08                      | 0.08 | 0.08 | 0.08 |
| Penicillin G      | 2.50                           | 0.003                     | 0.005 | 2.50 | 0.005 |
| Chlortetracycline | 0.31                           | 0.16                      | 0.08 | 0.08 | 0.08 |
| Neomycin          | 2.50                           | 0.08                      | 0.04 | 0.62 | 0.02 |
| Oxytetracycline   | 0.16                           | 0.08                      | 0.16 | 0.08 | 0.16 |
| Nitrofurantoin    | 1.25                           | 0.62                      | 2.50 | 1.25 | 2.50 |
| Erythromycin      | 0.62                           | 0.02                      | 0.04 | 0.04 | 0.02 |
| Novobiocin        | 0.31                           | 0.02                      | 0.08 | 0.08 | 0.16 |
| Bacitracin*       | >10.00                         | 0.004                     | >5.00 | 0.31 | 0.004 |

* Incubation time in hours. Minimum concentration detected in micrograms per disc.

*b* Units per disc.
Table 3. Effect of incubation temperature on sensitivity of B. megaterium to 17 inhibitory compounds when tested in Mueller-Hinton agar

| Inhibitor          | Incubation temp (C) |
|--------------------|---------------------|
|                    | 25 (12)             |
|                    | 32 (8)              |
|                    | 35 (7)              |
|                    | 37 (5)              |
| Sulfamethazine     | 0.16                |
| Sulfamethizole     | 1.25                |
| Sulfamerazine      | 0.16                |
| Sulfathiazole      | 0.16                |
| Sulfadiazine       | 0.16                |
| Sulfisomidine      | 0.16                |
| Sulfisoxazole      | 0.16                |
| Sulfanilamide      | 0.31                |
| Tetracycline       | 0.31                |
| Penicillin G       | 0.005               |
| Chlorotetracycline | 0.04                |
| Neomycin           | 0.02                |
| Oxytetracycline    | 0.16                |
| Nitrofurantoin     | 1.25                |
| Erythromycin       | 0.02                |
| Novobiocin         | 0.08                |
| Bacitracin*         | 0.08                |

*In incubation time in hours.

In general, the sensitivity of the procedure increased with increased incubation temperature, and 37°C was the temperature of choice.

Standard Methods for the Examination of Dairy Products recommends that all milks be heated to 82°C for 2 to 5 min to avoid reporting false-positives resulting from natural inhibitory substances in raw milk. Since this is part of the testing procedure and would affect apparent sensitivity if any of the inhibitors were sensitive to heat, all 17 inhibitors under study were assayed in milk before and after heating when incubated at 25, 32, 35, and 37°C. In no case was the apparent sensitivity of the test changed by more than one step in a twofold dilution series after the milk containing the inhibitors had been heated. This variation is normal for the procedure when used for repetitive testing of milk containing an inhibitor. Unlike the results of Marth, Alexander, and Hussong (2) in studies of the effect of heating milk on apparent sensitivity of assay, the heating technique of 82°C for 3 min had no effect on penicillin. This may be due to the heating techniques used since Marth et al. steamed their test milks for 7 min before testing.

With penicillin, penicillinase discs are used for identification in that the antibiotic is inactivated by the enzyme. Similarly, we tested 0.5-inch (12.7-mm) discs impregnated with 50 μg of p-aminobenzoic acid for their usefulness in identifying an inhibitor as a sulfa drug. These discs were made by adding the acid in aqueous solution to the disc, followed by drying the disc at 40 to 44°C. These discs inactivated the inhibitory properties of all sulfa drugs studied in concentrations of at least 5 μg of sulfa derivative per disc. Accordingly, we believe this technique is useful in identifying an unknown inhibitor as a sulfa drug.

From the alternatives studied, we believe the following to be most useful for the detection of sulfa drugs and antibiotics in milk: (i) add B. megaterium ATCC 9855 spores to Mueller-Hinton agar at 50°C to give a final spore concentration of 5 × 10^4 per ml of agar; (ii) dispense 4 ml of inoculated agar into a flat-bottom petri dish of about 90-mm inside diameter and agitate the dish so that the agar will cover the dish surface; (iii) allow agar to solidify on a level surface; (iv) touch edge of 0.5-inch (12.7-mm) filter disc to milk sample to wet disc by capillary action, and place disc on surface of inoculated agar; (v) incubate plates at 37°C for 4 to 5 hr; (vi) examine for zones of inhibition; and (vii) identify zones using conventional techniques as described in Standard Methods for the Examination of Dairy Products (1) including heating the milk at 82°C for 2 to 5 min to detect natural inhibitors. Discs containing p-aminobenzoic acid may be used to identify inhibition from sulfa drugs.

We believe that the substitution of the B. megaterium-Mueller-Hinton procedure for the one now in common use for testing milks offers the advantage of sensitivity to sulfa derivatives and to bacitracin. This is accomplished without significant change in sensitivity to the other antibiotics tested and without making the test procedure more difficult to perform. For these reasons, we feel that the procedure described merits consideration as a standard method for the regulatory testing of antibiotics and sulfa drugs in milk.

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