A RAPID DISC ASSAY METHOD FOR DETECTING PENICILLIN IN MILK

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NATIONAL SURVEY FINDINGS

Four nationwide surveys to determine the incidence of antibiotic residues in fluid milk have been conducted by the Food and Drug Administration[1, 3]. Penicillin was detected in 3.2 per cent of the samples tested in the 1954 survey, 11.6 per cent in 1955, 5.9 per cent in 1956, and 3.7 per cent in 1959. In these surveys an assay procedure requiring 16 to 18 hours was used[4]. Obviously, for regulatory purposes such a method is too time consuming to be of use in preventing the movement of adulterated milk in interstate commerce. Therefore, there is a need for a rapid method to assist regulatory agencies and the dairy industry in detecting the presence of penicillin in milk.

OTHER TEST PROCEDURES

Several microbiological methods have been developed for the detection of penicillin in milk. The overnight test previously mentioned is a cylinder-plate method using *Sarcina lutea* as the test organism[4] and it is sensitive enough to detect the presence of 0.005 unit per ml. Silverman and Kosikowsky[5] employed a disc assay method with Bacillus subtilis as the test organism and were able to detect 0.1 unit per ml. in 4 to 6 hours. Mattick et al[8] utilized the inhibitory effect of penicillin on the growth of *Staphylococcus aureus*, and by measuring the decreased production of nitrite from nitrate they were able to detect 0.1 unit per ml. Slatter[9] and Pital et al[10] described tests for the detection of penicillin in milk based upon its interference with the microbial reduction of resazurin. These test swere insensitive to concentrations of antibiotic below 0.1 unit per ml. Shahani and Badami[11] described a resazurin disc assay method which could detect 0.064 unit per ml. in 2 to 3 hours.

A "physical development" method described by Goyan et al[6, 7], is reported, in which a seeded plate was impregnated with silver nitrate, exposed to light, and finally subjected to a technique of physical development essentially equivalent to development of a latent image on a photographic emulsion. In our experience, this procedure neither shortened the incubation time nor increased the sensitivity of the test. Cerny and Morris[12] described a test that in 8 hours could detect 0.01 unit per ml.

In the methods described above, either the incubation time is relatively long, the test is insensitive to low concentrations of penicillin or the method is not readily adaptable to field testing. This report describes a simplified method which can detect the presence of 0.05 unit of penicillin per ml. of milk in 2-½ hours. The procedure is such that would permit a dairy technician to trace the source of milk containing penicillin by carrying a portable incubator and refrigerator in a car or truck, testing a milk sample on the farm, and obtaining results within 2½ hours.

PROCEDURE

CULTURE MEDIA. For carrying the test organism and for performing the assay, use a medium of the following composition (medium #1): Peptone 6 Gm., pancreatic digest of casein 4 Gm., yeast extract 3 Gm., beef extract 1.5 Gm., dextrose 1 Gm., agar 15 Gm., and distilled water to make 1,000 ml. The final pH after sterilization is 6.5 to 6.6. For preparing the test suspension use a medium of the same composition as medium #1 except that it contains in addition 300 mg. of $MnSO_4$. H_2O per liter (medium #2).

WORKING STANDARD. Dilute the penicillin working standard with sterile 1 per cent phosphate buffer pH 6.0 (2.0 Gm. dipotassium phosphate and 8.0 Gm. monopotassium phosphate per liter) to make a stock solution containing 100 units per ml. This stock solution may be used for no more than 2 days when stored at 15 C or less.

PREPARATION OF SAMPLE. The sample is undiluted milk.

PREPARATION OF TEST ORGANISM. The test organism is *Bacillus subtilis*, ATCC 6633. Maintain the test organism on nutrient agar medium #1 and transfer to a fresh slant every month. Inoculate a fresh slant of agar medium #1 with the test organism and incubate at 37°C for 16-24 hours. Wash the culture from the slant with sterile physiological saline onto the surface of a Roux bottle containing 300 ml. of medium #2. Incubate 37°C for 5 days. Wash the resulting growth from the agar surface with 50 ml. of sterile physiological saline, centrifuge, and decant the supernatant liquid. Reconstitute the sediment with sterile physiological saline and heat-shock the suspension by heating for 30 minutes at 70°C. Maintain the spore suspension at approximately 15°C. This spore sus-

pension will keep for several months. Add 2 ml. of the spore suspension to each 100 ml. of agar medium #1 for the test.

PREPARATION OF PLATES. Add 10 ml. of inoculated agar (prepared as above) to each 20x100 mm. Petri dish. Plastic dishes may be used. Distribute the agar evenly in the dishes, cover with porcelain covers glazed only on the outside and allow to harden. Store at approximately 15°C for not less than three, nor more than five days. Remove each Petri dish as needed and use within 15 minutes.

DISCS. Use round, white paper discs with a diameter of ¼ inch. The paper used for the disc should be Schleicher and Schuell No. 740-E, No. 470-W, or No. 470, or paper of comparable grade, absorption, performance qualities, and purity. No. 740-E is available as discs already punched to the recommended size. The others are available in sheets from which discs may be punched.

ASSAY PROCEDURE. At the time a new spore suspension is prepared test plates should be run to determine its maximum sensitivity to penicillin. In our laboratories the maximum sensitivity has been 0.05 unit per ml. of milk.

Use a pair of forceps and dip a paper disc completely into the sample to be tested. Shake the disc to remove any excess milk and place on the surface of the agar, touching the disc gently with the tip of the forceps to ensure proper contact of the disc with the agar. Place the discs so that they are at least 20 mm. apart when measured from center to center to avoid overlapping of zones. In this manner many samples may be tested on the same Petri dish. Control discs may be prepared by dipping the discs in milk containing 0.05 unit of penicillin per ml. These may be dried and used for several days if stored in tightly stoppered vials. Incubate at 37 C for approximately 2-½ hours and then examine for any sign of inhibition of the test organism. At this time the test organism will have grown sufficiently so that if penicillin is present in milk at a concentration of at least 0.05 unit per ml., a zone of inhibition will be discernible. The plate should be held at various angles to be light source until the light is at the proper angle for best observing the zone of inhibition.

If a zone of inhibition is obtained, determine if the activity is due to penicillin as follows:

Add 0.05 ml. (approximately one drop) of penicillinase concentrate to a 5 ml. aliquot of the milk sample and shake well. Prepare three discs from the penicillinase treated sample and three discs from an untreated aliquot of the sample. Place all six discs on one plate, proceeding as described under "Assay procedure." A zone around the discs dipped in untreated milk, but no zone around the discs dipped in penicillinase treated milk, is a positive test for penicillin.

EXPERIMENTAL

Samples of milk were prepared containing graded concentrations of penicillin. The minimum incubation period at 37°C required to observe sufficient growth of the test organism, in turn making it possible to see a zone of inhibition, was 2-½ hours, and the maximum sensitivity of the organism was 0.05 unit per ml. A control disc was placed on each Petri dish. Modifications of methods previously described such as the use of resazurin were attempted. Indicators had a tendency to mask the growth of the test organism, thus making it more difficult to detect a zone of inhibition. In all instances, a zone could be observed more readily if no indicator or dye was used. Preincubation of the inoculated dishes for ½ or 1 hour at 37°C was also attempted in order to decrease the time required for the test. However, while growth of the test organism was noted in a shorter time, the test was not as sensitive.

DISCUSSION

The rapid method described in this paper is only one-tenth as sensitive as the overnight procedure.[4] However, it should be of value for detecting in a minimum amount of time those milk samples which contain relatively high concentrated of penicillin (greater than 0.05 unit per ml.). It is estimated that in the nationwide surveys of 1954, 1955, 1956, and 1959, the rapid method would have detected penicillin in 33, 7, 16, and 58 per cent, respectively, of the samples which were reported as containing penicillin when tested by the overnight procedure.

SUMMARY

A simplified and rapid method is described for detecting the presence of penicillin in milk in concentrations as low as 0.05 unit per ml. The method may be used by the dairy industry with a minimum of equipment.

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AMENDMENT TO SUPPLEMENT No. 3 TO THE 3-A SANITARY STANDARDS FOR FITTINGS USED ON MILK AND MILK PRODUCTS EQUIPMENT AND USED ON SANITARY LINES CONDUCTING MILK AND MILK PRODUCTS, APRIL 26, 1955

Serial #0803-A
Formulated by
International Association of Milk and Food Sanitarians
U. S. Public Health Service
The Dairy Industry Committee

November 1959

In keeping with the provisions of the 3-A Sanitary Standards for Sanitary Fittings and Connections Used On Milk and Milk Products Equipment, this supplement incorporates the following paragraphs covering "Special Sanitary Fittings" into the standards:

SPECIAL SANITARY FITTINGS

As 3-A Sanitary Standards are not intended to limit individual ingenuity, this supplement sets forth a basis for the approval of special sanitary fittings to meet specific applications where standard 3-A Sanitary Fittings designs are not applicable.

Where special sanitary fittings are required and interchangeability with respect to face to face, or centerline to face dimensions is not important, the following conditions must be met:

These special fittings must qualify with respect to material, finish, construction, thread dimensions (if threads are used), and use of gaskets as set forth for approved 3-A fittings in the standard to which this statement is a supplement. All product contact surfaces of such fittings shall be accessible for cleaning and inspection. All internal angles shall have radii of not less than 1/16-inch, except gasket recesses and grooves in which all sharp corners shall be avoided.

This amendment becomes effective upon publication.