A SIMPLE FIELD TEST FOR THE DETECTION OF INHIBITORY SUBSTANCES IN MILK SUPPLIES

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SUMMARY

A modification of a previously reported non-acid test uses only simple equipment which is normally available at factories for methylene blue testing. The method depends upon the slower reduction of methylene blue by *Streptococcus thermophilus* during incubation at 45°C. It detects penicillin at a concentration of 0·02 I.U./ml and is sensitive also to oxytetracyline and chlortetracycline.

I. INTRODUCTION

Even if dye-markers were included in penicillin preparations for use in udder infusion, they would not be entirely satisfactory as indicators of inhibitory substances, as their secretion precedes that of the residual quantities of antibiotics normally left in the udder and which affect cheese starter activity in cheese manufacture. There would still be a need for a rapid simplified field test for the detection of inhibitory substances or antibiotics in milk.

Several of the factory tests proposed for the detection of penicillin (Berridge 1956; Keogh 1961; Wright and Tramer 1961) utilize substances or reagents which are not normally available at the factory. Cox (1934) developed a non-acid test using normal cheese starter cultures in a modified methylene blue reductase test on each pasteurized milk supply. A similar method involving methylene blue reduction was reported by Feagan and Bray (1961) during the course of this work.

The test detailed below involves the reduction of methylene blue using *Streptococcus thermophilus* TS2.

II. METHODS

(a) Inhibitory Test

Stock Cultures.—The culture used was a strain of *S. thermophilus* TS2 which was obtained freeze-dried from the Commonwealth Scientific and Industrial Research Organisation. Two starter cultures—HP, a strain of *S. cremoris,* and C2, a strain of *S. lactis*—were used in comparative tests.

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Penicillin-free skim-milk was used for the maintenance of the cultures. Carbonated litmus milk was inoculated weekly and refrigerated after incubation at 37°C for 24 hr. Sterile skim-milk was inoculated with 1 per cent. inoculum from the above refrigerated sample and incubated at 37°C for 24 hr before use in the test.

Procedure.—Initial trials were made using the non-acid test of Cox (1934), which includes pasteurization of the sample and incubation at 37°C. This was later modified by using TS2 starter instead of a normal *S. lactis* or *S. cremoris* culture and incubating the test at 45°C. The method finally adopted was as follows:—Ten millilitres of the milk to be tested was poured into sterile test-tubes, and 1 ml standard methylene blue solution and 2 drops of TS2 culture added, using 3/16 in. sterile glass tubing. The tubes were incubated in the 45°C water-bath for 2½ hr without inversion.

(b) Disc-assay Method

The disc-assay method for the detection of penicillin was substantially that used by Naylor (1960), with minor modifications in the methods used for obtaining the standard suspension of the test organism.

III. RESULTS

(a) Modifications of the Non-acid Test

In the first tests, 3 drops of starter were added from pipettes with the same bore dimensions to 20 ml milk containing 0·05 I.U. per ml penicillin. Starters used were HP, C2 and TS2. It was found that there was no variation in reduction time among tubes containing the same culture.

Milk samples were then prepared containing amounts of a standardized penicillin preparation (C.S.L. benzyl penicillin subsidiary standard) ranging from 0·01 to 0·04 I.U. per ml. Milk without penicillin and with each concentration of penicillin was then inoculated with varying amounts, ranging from 3 to 8 drops, of each of the three cultures. Set out in Table 1 are the results obtained in the milk containing 0·04 I.U. per ml.

TABLE 1

EFFECT OF VARIATION IN AMOUNT OF STARTER INOCULUM ON REDUCTION TIME (IN HOURS) AT 37°C

Starter	No. of Drops		
	3	5	8
TS2	>4½	>4½	>4½
C2	2½	1½	1
HP	2½	1½	½

It was found that variation in the amount of inoculum did not appreciably affect the reduction time when TS2 was used, but when HP and C2 were used the reduction time decreased as the amount of starter was increased.

One disadvantage of using normal starter cultures was that a standard drop size was necessary. It was also very difficult to appreciate the gradation of colours of the control milks with penicillin concentrations ranging from 0·01 to 0·05 I.U. per ml. On the other hand, with TS2 a definite blue colour was found to persist with concentrations of 0·02 I.U. per ml and over.

As 10-ml methylene blue tubes are standard equipment in factories, the sensitivity of the test using 2 drops of TS2 culture in 10 ml milk was determined. Incubation was at 37°C. These results are shown in Table 2.

TABLE 2

SENSITIVITY OF TEST USING 2 DROPS OF TS2 IN 10 ML MILK AT 37°C

No. of Samples	Penicillin Concentration (I.U./ml)	Reduction Time (hr)
3	nil	3
3	0·01	3
3	0·02	>3
3	0·03	>3

Of 196 samples tested with this method and the disc-assay method, 7 were found to contain more than 0·01 I.U. in the disc-assay and all were positive by the methylene blue reduction method.

(b) Use of Reduction Test at 45°C

As *S. thermophilus* grows rapidly at 45°C, the value of reduction tests at this temperature was determined. Wright and Tramer (1961) used growth of *S. thermophilus* at 44-45°C in their test for penicillin, using 2,3,5-triphenyltetrazolium chloride (TTC), and Feagan and Bray's (1961) reductase test is carried out at 44°C. The use of this higher temperature excludes the need for laboratory pasteurization of the milk, as the influence of the normal milk flora would be negligible at this temperature.

A series of milks with added penicillin was tested to determine the sensitivity of the test at 45°C. The results are shown in Table 3.

TABLE 3

SENSIVITY OF TEST USING TS2 AND INCUBATION AT 45°C

No. of Samples	Penicillin Concentration (I.U./ml)	Reduction Time (hr)
6	nil	2½
6	0·01	3
6	0·02	>5

This method was used to test 1081 samples and comparisons were made with results obtained in the disc-assay method. The latter method showed that 36 samples (3·3 per cent.) contained 0·02 I.U. or more of penicillin, and 34 (3·14 per cent.) of these were found to be positive by the modified methylene blue method. The results of these tests are shown in Table 4.

TABLE 4

COMPARISON OF RESULTS OF SAMPLES TESTED BY DISC-ASSAY AND INHIBITORY METHODS

Concentration of Penicillin (Disc-assay Method) (I.U./ml)	No. of Samples	No. Positive in Modified Methylene Blue Test
0·01	7	1
0·02	8	6
0·03	9	9
0·04	5	5
0·05	5	5
0·06	1	1
0·07	—	—
0·08	3	3
0·1 and >0·1	5	5

(c) Sensitivity to Other Antibiotics

Investigations were made of the sensitivity of the test both at 37°C and 45°C to other antibiotics. It was found to detect additions of the antibiotics down to approximately 0·5 μg/ml of "Aureomycin" (chlortetracycline) and to approximately 0·2 μg/ml of "Terramycin" (oxytetracycline).

IV. DISCUSSION

As the test described detects antibiotic substances other than penicillin, it has greater value than the Naylor disc-assay method, which is intended primarily for detecting penicillin. The sensitivity of the reductase method, as of other tube methods, is not so great as that of the disc-assay but the levels detected are adequate for routine purposes.

For the detection of antibiotics, the method should be used on fresh morning's milk. It was shown by Cox (1934) that poor quality milks having a reduction time of less than 2 hr could contain inhibitory substances due to the growth of organisms in the milk. While the test recommended determines the presence of inhibitory substances as a whole, to obviate the possible effects of substances

due to bacterial growth such as nisin, milks with normal methylene blue reduction times of less than 2 hr should not be used. In the present investigations it was also found that the sensitivity of the test for antibiotics using raw milks was lowered if the samples were slightly stale.

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