VARIABLE FACTORS IN THE NEW TEST FOR PENICILLIN IN MILK

LLOYD D. WITTER AND STEWART L. TUCKEY

Department of Food Technology,

University of Illinois, Urbana

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Fluid milk plants throughout the country are now testing producer's milk for the presence of penicillin. The testing procedure in most common use is that of Arret and Kirschbaum (2) since this method is the one being employed by the Food and Drug Administration and would be the basis of action by them. As pointed out by Johns (5) this testing procedure offers no advantages over the modified Difco method given in *Standard Methods for the Examination of Dairy Products* (1) and has several disadvantages. The Arret and Kirschbaum procedure recommends that the test be run at 37°C. and the results read after 2½ hours incubation. This specified temperature is not commensurate with incubators found in most dairy laboratories and while rapid tests are desirable it is usually, unnecessary to know the results this quickly when surveying a producer's milk. It was further recommended that plates be stored for not less than three days nor more than five days before being used to perform the assay. This recommendation would require careful planning ahead by the dairy laboratory to run the test and avoid waste.

Most dairy laboratories must rigorously follow the specifications of any new test since they do not have the time to determine other conditions that might give equivalent results. This study was undertaken in an effort to evaluate the results obtainable by the Arret and Kirschbaum procedure when the temperature for storing plates, time of storing plates, temperature for the test, and time of reading the test were modified.

PROCEDURE

The Arret and Kirschbaum procedure was followed as far as possible, but modified to show the effect of several variable conditions and modified to conform with the manner in which it would normally be run in a dairy control laboratory.

Bacto Antibiotic Medium 1 was used for performing the assay and this same medium plus 0.03 per cent manganese sulfate was used for preparing the spore suspension. The spore suspension was prepared in the prescribed manner using a Bacto Standardized Spore Suspension (0453) of *Bacillus subtilis* ATCC No. 6633 as the inoculum. The spore suspension was initially heat-shocked, although the effect of heat-shocking (to produce a maximum germination of spores) is known to rapidly disappear with storage (3, 4). Therefore, the spore suspension was again heat-shocked by adding it to the agar medium as soon as it was melted. The hot seeded agar was allowed to cool for 10 to 15 minutes at room temperature and then the plates were poured. The prepared plates contained between 6 and 10 ml. of agar and glass rather than the recommended porcelain petri dish covers were used. A separate plate was prepared for each combination of variables, which included: (*a*) storage of from one to ten days or used immediately, (*b*) storage at a temperature of 2°, 5°, 10°, or 15°C., (c) temperature for performing the assay of 32°, 35°, or 37°C., and (*d*) reading of the test plates at 2½, 4, or 6 hours. Each plate was spotted with a 0.25 in. disc (Bacto 0.25 in. Sterile Blank Concentration Disks) and a 0.5 in. disc (Schleicher and Schuell #740-E), which had been dipped in a freshly prepared solution of penicillin (Penicillin G-sodium, working standard WO 2171, Eli Lilly) containing 0.1 unit per ml. Preparation of plates for storage and for assay was so arranged that all variables were read at essentially the same time (over a 30-minute period) so that the zones formed could be compared with one another.

RESULTS AND DISCUSSION

Although the variables under which the test for penicillin was run in this study were somewhat interdependent, for the sake of clarity each variable will be considered separately. The results of this study are shown in Table 1.

Temperature of Storage

The reliability of the assay test was relatively independent of the temperature at which the plates were stored. Although faster results appeared to be obtainable on plates stored at 15°C., this storage temperature presented several disadvantages; facilities for storing at this temperature are not normally found in a dairy laboratory and the plates may be kept for only a few days until growth of the test organism is too extensive to permit their use in testing. Of the refrigeration temperatures (2°, 5°, and 10°C.) slightly better results were obtained at the lower temperatures.

Time of Storage

Good results were obtained on plates which were

TABLE 1—EFFECT OF VARIOUS FACTORS ON THE DEVELOPMENT OF READABLE ZONES OF INHIBITION IN A DISC ASSAY PROCEDURE FOR THE QUALITATIVE DETECTION OF PENICILLIN (0.1 UNIT PER ML.). BOTH 0.25 IN. AND 0.5 IN. DISCS WERE TESTED AND GAVE IDENTICAL RESULTS.

Conditions of storage of plates		Time of incubation of plates (hours)							
		2½			4			6	
Temperature (°C.)	Time (days)	Temperature of incubation of plates (°C.)							
		32	35	37	32	35	37	32	35
15	2	+	+	+	++	++	++	++	++
	1	-	+	-	+	++	+	++	++
10	9	-	-	-	−	−	−	+	++
	8	-	-	-	−	+	+	+	++
	7	-	-	+	+	++	++	++	++
	6	-	-	-	−	−	−	−	++
	5	-	-	-	−	−	−	−	++
	4	-	-	-	−	+	+	+	++
	3	-	-	-	−	−	−	−	++
	2	-	-	-	−	−	−	−	NT
	1	-	-	-	−	−	−	−	++
5	10	-	-	-	−	++	++	++	++
	9	-	-	-	−	−	−	−	++
	8	-	-	-	−	−	−	−	++
	7	-	-	-	+	++	++	++	++
	6	-	-	-	−	+	−	++	++
	5	-	-	-	−	−	+	−	++
	4	-	-	-	++	++	++	++	++
	3	-	-	-	−	−	−	−	++
	2	-	-	-	+	+	−	++	++
	1	-	-	-	++	++	−	++	++
2	6	-	-	-	+	++	++	++	++
	5	-	-	-	−	−	−	−	+
	4	-	+	+	+	++	++	++	++
	3	-	-	-	−	++	−	NT	++
	2	-	-	-	+	++	+	+	++
	1	-	-	-	+	++	++	++	++
No storage	0	-	++	+	+	++	++	++	++

− = No visible growth of Bacillus subtilis—no zone.
+ = Slight growth of Bacillus subtilis—zone difficult to see.
++ = Good growth of Bacillus subtilis—zone easy to see.
NT = Not tested.

used immediately after preparation and there was no evidence to support the "not less than three days" storage recommendation of Arret and Kirschbaum (2).

While the time of storage did not appear to be an important variable there was a definite upper limit of storage primarily dependent upon the degree of dehydration of the plates. Only portions of some of the plates stored for nine days at 10°C. were in satisfactory condition for running the assay due to dehydration of the agar. The upper limit, however, as shown by the results, was not limited to the "not more than five days" recommendation of Arret and Kirschbaum. Plates stored six to ten days gave as satisfactory results as those stored from one to five days. Furthermore, three plates with porcelain covers were stored at 2°C. for 66 days and gave readable positive tests in approximately six hours at 37°C.

Temperature of Incubation

Whether a given incubation temperature for performing the assay was satisfactory or unsatisfactory was dependent upon how rapidly one wished to know the results. Presumably, the most desirable temperature, however, would be that at which plates should be incubated to give a positive zone in the shortest time. The results in Table 1 showed that equally rapid results were obtained at either 35° or 37°C., but that the test was substantially slower at 32°C.

Incubators at 37°C. are not normally found, while incubators at 35°C. are commonly found in dairy laboratories. Since equally satisfactory results were obtained at either temperature, a temperature of 35°C. would seem to be the logical choice of temperature for testing penicillin in milk in the dairy laboratory.

Time of Incubation and Reading of Assay

Arret and Kirschbaum observed that by using their procedure the minimum incubation time at which it was possible to see a zone of inhibition was 2½ hours. The results of this study showed that on only a very few plates spotted with penicillin containing discs was it possible to see a zone of inhibition in this minimum time of 2½ hours incubation. Of the 28 plates assayed at 37°C. only four had sufficient growth of *Bacillus subtilis* in 2½ hours to give a detectable zone. Nine of these plates followed the limitations imposed by the Arret and Kirschbaum procedure and only one of the nine gave a detectable zone in 2½ hours. These same figures held true for plates which were incubated at 35°C. Quite simply, 2½ hours was an insufficient time to recommend reading the plates, although a few were readable in that time of incubation.

After six hours incubation at 32°C. approximately one-half of the plates had readable zones of inhibition, which were roughly comparable to plates incubated for four hours at either 35° or 37°C. Therefore, if the test were to be run at 32°C. a reading time greater than six hours would normally be required. After six hours incubation at 35°C. all of the plates had readable zones of inhibition — this would probably be true of plates incubated for six hours at 37°C., but this was not tested.

After recording the results in Table 1 all of the plates were allowed to remain overnight at room temperature. After this further incubation period all of the plates had easily readable zones of inhibition. Johns and Berzins (*6*) experienced and pointed out that on overnight incubation large surface colonies might obscure the zones when using 0.25 in. discs. In this laboratory no disadvantage was found in incubating plates overnight when 0.25 in. discs impregnated with a penicillin solution containing as little as 0.05 units per ml. were used.

Size of Disc

At the concentration of penicillin used in these tests (0.1 unit per ml.) equivalent positive results were obtained with either 0.25 in. or 0.5 in. discs. The sensitivity of 0.25 in. and 0.5 in. discs, however, was separately tested. The 0.5 in. discs gave positive zones when dipped in samples containing 0.03 unit per ml., but not in samples containing 0.01 unit per ml. of penicillin, while 0.25 in. discs detected samples containing 0.05 unit per ml., but not samples containing 0.03 unit per ml. These results confirmed those of Johns and Berzins (*6*) who reported a greater sensitivity for the 0.5 in. disc. Tests run with the 0.5 in. discs, however, are slightly more expensive and slightly less convenient than the tests using the 0.25 in. discs, since fewer discs can be placed on a single plate. Each dairy technologist might weigh these factors in making his choice of disc size.

Volume of Agar

The greater the depth of agar in a plate the less sensitive the disc assay test becomes and for a given concentration of penicillin the less the zone of inhibition will be. Hence, to assure equivalent zone sizes for equivalent concentrations of penicillin, the agar must be accurately measured into each plate, the plates must have flat bottoms, and the agar must be allowed to harden on a level surface to assure a uniform and constant depth of agar in each plate. The purpose of the disc assay test discussed in this paper, however, is to determine the presence or absence of penicillin in milk samples at a concentration level of 0.05 unit per ml. or higher. To fulfill this purpose the depth of agar in a plate need not be uniform and constant, but it must be small enough to assure a readable zone of inhibition when samples containing 0.05 unit per ml. of penicillin are tested. To observe the limitations of agar depth on this test, 6, 10, 15, and 20 ml. of agar were accurately pipetted into quadruplicate assay test plates observing the above listed precautions. On these plates 0.25 in. discs dipped in samples containing 0.05 unit per ml. of penicillin gave average zones of inhibition of 13, 10, 8, and 0 mm respectively and when dipped in samples containing 0.1 unit per ml. of penicillin the average zone sizes were 16, 13, 10, and 9 mm respectively. Hence, if 10 ml. of agar or less is used per plate the presence or absence of penicillin in samples containing 0.05 unit per ml. or higher can be detected using 0.25 in. discs without undue concern about a lack of uniform depth of agar giving a negative test.

CONCLUSIONS

The penicillin assay test was substantially independent of the refrigeration temperature at which the plates were stored prior to use, although dehydration of the plates was less at lower temperatures.

No evidence was found to support the recommendation that plates be stored at refrigeration temperatures for not less than three days nor more than five days before being used to perform a penicillin assay. Satisfactory results were obtained on plates used immediately after preparation and on plates stored over two months. Plate storage, however, was limited by dehydration.

Equally satisfactory results were obtained with plates incubated at 35°C. or 37°C. Since 37°C. incubators are not normally found in dairy laboratories, 35°C. would be the more realistic temperature to recommend for incubating plates when testing for penicillin in milk.

Zones of inhibition were observed on a very few plates in 2½ hours, on approximately one-half of the plates in four hours, and on all of the plates in six hours when incubated at 35°C. Hence, to assure visible zones of inhibition, plates should be incubated from four to six hours, or until growth is apparent.

The above suggested changes make the Arret and Kirschbaum method less restrictive, more applicable to dairy laboratory procedures, and more likely to detect all positive samples. In fact, these changes, with minor exceptions, renders the method described by them indistinguishable from the modified Difco method suggested in the *Standard Methods for the Examination of Dairy Products.*

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FEDERAL REGULATION IN THE FIELD OF IDENTITY, QUALITY AND SANITARY STANDARDS FOR MILK AND MILK PRODUCTS[1]

CHARLES M. FISTERE

General Counsel for the Dairy Industry Committee,

Continental Building, Washington, D. C.

At one time or another most everyone has undertaken to put a jigsaw puzzle together. It is, as a matter of fact, somewhat of a minor satisfaction when, after fitting together a few hundred oddly shaped pieces, there finally emerges an integrated picture. It might be a landscape or pastoral scene or it might be a map of a foreign land, but in any event, the result represents a whole unified pattern.

An investigator who undertakes to assemble the few hundred oddly shaped pieces which represent federal activity in the field of identity, quality and sanitary standards of dairy products will not finish, I can assure you, with any unified integrated pattern – much less a pastoral scene.

This is not to say there is not a good deal of dovetailing between the programs of the several federal agencies involved. As a matter of fact, despite the fact of duplicate authorizations in a number of fields, there has been a large measure of cooperation – both inter-agency and between agencies and industry. This has resulted in less conflict than one would suspect, since the three agencies of the federal government have responsibilities in the field of standards for dairy products.

The subject assigned is of treatise magnitude. In a paper of appropriate length for a meeting such as this it will be attempted to sketch the outlines of the subject matter in three ways. First, to outline the enabling laws; second, to briefly review what has been done under these laws; and third, to discuss some similarities, differences and areas of possible duplication.

It seems to me that the proper starting point is with the federal statutes involved.

Even though three agencies of the federal government are concerned with standards for dairy products, one of the first distinctions which becomes apparent is the different underlying purposes on which the authority is grounded. While it is perhaps an over-simplification, and although there is some overlap, I think it fair to say that standards of identity established pursuant to the Federal Food, Drug and Cosmetic Act are designed to prevent the perpetration of economic fraud upon consumers. The purpose of model ordinances and codes of the Public Health Service is, to be sure, the preservation of the public health. Whereas, the purpose underlying the activity of the Department of Agriculture in the field

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