Evaluation of a Rapid Test for Antibiotic Residue in Milk Using Spores of *Bacillus stearothermophilus* var. *calidolactis*[1]

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ABSTRACT

Eleven analysts tested contaminated reconstituted (1:10) dry milk powders for penicillin residues using spores of *Bacillus stearothermophilus* var. *calidolactis* (Delvotest P method). Three types of responses were noted: positive, negative, and questionable. Prediction equations indicated that 95% of the time, analysts unfamiliar with the technique could detect positive results if penicillin concentrations in samples were 0.010 unit/ml or higher and positive and questionable results if the penicillin concentrations were 0.008 unit/ml. Increasing the reconstitution ratio from 1/11 to 1/4 increased the chances of detecting penicillin in milk powder. Penicillinase added to reconstituted penicillin-contaminated milks in all instances produced negative responses.

Many methods have been described for microbiological methods for detection of antibiotic residues in milk (review by Marth, *10*). Milk-impregnated discs on agar plates seeded with spores of either *Bacillus subtilis* (*2, 10, 18*) or with *Bacillus megaterium* (*14*) have been used.

Thermophilic bacteria have been used for rapid tests (2 1/2 h). Berridge (*3*) employed *Streptococcus thermophilus*. Igarashi et al. (*6*), using *Bacillus stearothermophilus*, showed this organism to be suitable for detecting antibiotics in raw milk by either the triphenyltetrazolium (TTZ) reduction method or by a disc assay. Galesloot and Hassing (*5*) indicated that as little as 0.0025 unit penicillin/ml of milk could be detected with *B. stearothermophilus* by a paper disc method. Kabay (*8*), using a cylinder cup assay, showed the organism to be sensitive to 14 antibiotics and several chemical preservatives; the minimum concentration of penicillin detected was 0.01 unit/ml. Terplan (*16*) and Jacquet and Riquier (*7*) used *B. stearothermophilus* var. *calidolactis* as the test organism in a disc assay. The latter workers indicated that this test could detect 0.0025 unit penicillin/ml. They found the method lacking in reproductibility when used for quantitative assay and suggested that it would be practical only if rigidly standardized. Forschner (*4*), on the other hand, showed this organism to be sensitive to 0.004 unit penicillin/ml in a disc assay when incubated 3 h at 70 C and used it successfully to quantitate penicillin. Picmanova et al. (*13*) also obtained good results with this variety of *B. stearothermophilus*. Romond et al. (*15*) indicated that at least one source of potential error could arise from the presence of antibiotic producing bacilli in milk, especially if incubation was extended beyond 2 1/2 h.

Van Os et al. (*17*) described a variation of the *B. stearothermophilus* var. *calidolactis* method. They used ampules containing agar seeded with spores, which, when nutrients were added, allowed germination and outgrowth of the organism with acid production showing up as an indicator change from purple to yellow. When antibiotics were present the indicator remained purple. Gist-Brocades, Delft-Holland, developed this method commercially as Delvotest P. Packard et al. (*12*) compared the *B. subtilis* disc assay (*1*) with the Delvotest P method and detected more positive raw milk samples with the latter.

The study reported here was a collaborative test to evaluate the reproducibility and sensitivity of the Delvotest P method for detection of penicillin in milk.

MATERIALS AND METHODS

Samples and analysts

Five antibiotic-contaminated [(determined by the FDA using the *Sarcina lutea* assay (*11*)] non-fat dry milk samples were obtained by the test coordinator (CNH) from supplies seized by the USDA, together with two samples free of antibiotics. The contaminated powders were diluted 1/2 (1:1) and 1/4 (1:3) with the antibiotic-free powder. The original, nondiluted powders gave a positive (+) reaction in the laboratory of the coordinator, while 1/2 dilutions gave questionable (±) reactions, and 1/4 dilutions gave negative (–) reactions.

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Artificially contaminated powders were prepared by mixing finely ground procaine penicillin G, tetracycline • HCl, or neomycin sulfate with control milk powder. The antibiotic level of the most concentrated powder was adjusted to that claimed to be within the sensitivity of the Delvotest P method and dilutions of 1/2 and 1/4 were made. The three actual concentrations of penicillin, as determined by the *S. lutea* assay for the three preparations, were 0.0040, 0.0020, and 0.0015 unit/ml on a reconstituted basis. Tetracycline concentrations were determined by the method of Kramer et al. (*9*) on dry milk powders containing approximately 520 mcg/g. These were diluted with antibiotic-free powder to give reactions of +, ±, and − when diluted 1/11 with water; the actual assayed values were, respectively, 0.192, 0.089, and 0.058 mcg/ml on a reconsituted basis. Neomycin in the same final dilutions gave +, ±, or − reactions, and, when assayed by a cylinder cup method (Oostendorp, personal communication) contained respectively, 26.5, 19.0, and 9.0 mcg/ml on a reconstituted basis.

The original and two dilutions (1/2, 1/4) of the five contaminated powders, the artificially contaminated (penicillin, tetracycline, and neomycin) and control powders, together with Delvotest P kits and penicillinase solution (containing 30×10^6 Kershey units/ml, prepared by the FDA laboratories, Washington, D.C.) were sent to the 11 participating analysts in the states of Indiana, Kentucky, Louisiana, Minnesota, Missouri, Ohio, Oregon, Tennessee, Texas, and Wisconsin.

Reconstitution and assay

Analysts were instructed to thoroughly mix duplicate 1-g portions of powder with 10 ml of water, add 0.1 ml to the ampules, incubate 2 1/2 h at 64-66 C, and record the results. If the agar remained completely purple, a + reaction was to be recorded; if zones of yellow and purple were noted, the reaction would be ±; a completely yellow agar would indicate −. The remaining portions of the diluted powders were refrigerated; those showing a + reaction were tested within 2 days for penicillin by adding two drops penicillinase to room temperature-warmed samples; after 10 min the test was repeated. The samples showing a ± or a − reaction in the initial test were reassayed using 1 g of powders and 3 ml of water (1/4 dilutions).

RESULTS

Types of responses obtained

The numbers of +,±, and − responses obtained for each powder are in Table 1. The numbers of + responses of a total of 40 varied from five (analysts 7 and 8) to 16 (analyst 2) and the ± responses varied from three (analyst 2) to 18 (analyst 7). Negative results varied from 11 for analyst 11 to 28 for analyst 8. The two control powders, M and Z, gave no + and only one ± response. Those indicated as having a trace of antibiotics by the *S. lutea* assay (these were dilutions of powders known to be contaminated) gave four + responses, all from analyst 11, and 17 ± responses. Sample C showed only four + responses by the analysts, although originally it gave a + response in the coordinating laboratory. All + samples (of those known to contain penicillin(were tested for penicillin by the addition of penicillinase and became −.

Increase in sensitivity with 1/4 dilution

Milk samples originally exhibiting ± or − responses, when reassayed at 1/4 concentrations, showed that of 40 samples for which − responses were originally obtained, 16 (40%) remained −, 16 became ± and eight became +. Of 34 originally showing ±, in tests repeated at 1/4 levels, seven (21%) remained ± and 27 (79%) became +. The three samples showing a trace of antibiotic (samples P, R, and U) in the *S. lutea* assay gave an average of 96% + or ± responses in the 1/4 dilution but only 32% for the 1/11 dilutions. The + responses were 59% for samples diluted 1/4 as compared to 6% for samples diluted 1/11. The antibiotic-free powders, M and Z, showed no

TABLE 1. *Responses to penicillin-contaminated and control milk powders (1/11 dilution) using the Delvotest-P method*

Sample[a]	Penicillin (unit/ml)	Original Test[b]	Analyst 1	2	3	4	5	6	7	8	9	10	11	Total +	±	−
C	0.0040	+	−	−	+	+	−	−	±	±	−	−	±	4	6	12
B	0.0020	±	−	±	+	+	−	−	±	−	−	±	−	3	4	15
A	0.0015	−	−	−	−	−	−	±	−	−	−	−	−	0	1	21
J	0.0070	+	+	+	+	+	+	+	+	±	+	+	±	19	3	0
K	neg[c]	±	±	−	±	±	+	+	±	±	±	±	±	3	18	1
L	neg	−	−	−	−	±	±	−	−	−	±	±	−	0	8	14
N	0.0086	+	+	+	+	+	+	+	+	+	±	+	+	21	1	0
O	0.0052	±	±	+	+	±	±	±	+	−	±	−	+	10	9	3
P	trace[d]	−	−	−	−	−	−	±	−	−	−	−	±	1	3	18
Q	0.0058	+	+	±	+	±	±	±	+	±	−	±	+	9	12	1
R	trace	±	−	−	−	−	−	±	±	−	−	±	±	1	7	14
S	neg	−	−	±	−	−	−	−	−	−	−	−	±	0	3	19
T	0.0086	+	+	+	+	+	±	+	+	±	−	±	+	14	7	1
U	trace	±	−	±	−	−	−	±	±	−	−	±	+	2	7	13
V	neg	−	−	−	−	−	−	−	−	−	−	−	+	2	0	20
W	0.0084	+	+	+	+	+	+	+	+	+	+	±	+	20	2	0
X	0.0040	±	±	−	±	±	−	±	±	±	−	±	±	0	15	7
Y	neg	−	−	−	−	−	−	±	−	−	−	−	±	0	4	18
M	neg	−	−	−	−	−	−	−	−	−	−	−	−	0	0	22
Z	neg	−	−	−	−	−	−	−	−	−	−	−	−	0	1	21
		+	11	16	8	7	8	14	5	5	13	7	15	109		
	TOTALS	±	6	3	10	11	8	10	18	7	13	11	14	111		
		−	23	21	22	22	24	16	17	28	14	22	11	220		

[a]Grouped samples are dilutions of same artificially or naturally contaminated powders.
[b]Determined in coordinator's laboratory before samples sent to analysts.
[c]No zone in *S. lutea* assay.
[d]Zone in *S. lutea* assay but not in range of tests.

increase in + or ± responses. Samples K, L, S, V, and Y, which were contaminated but gave no zones of inhibition in the *S. lutea* assay, gave 16.3% and 60.8% + or ± responses when diluted 1/11 or 1/4, respectively.

Correlation of response with penicillin concentration

Responses of analysts, arranged in order of increasing penicillin concentration (Table 2), showed an apparent correlation with antibiotic levels of the milk. At concentrations of 0.0084 unit/ml or greater almost 100% of the responses were either + or ± (84% were +). Samples with lower content of penicillin gave fewer + results. The ± results were most numerous in the intermediate penicillin (0.004 to 0.0058 unit/ml) samples.

TABLE 2. *Effect of penicillin concentration on the percentage of + or + and ± responses in milk powders diluted 1/11*

Sample	Penicillin[a] unit/ml	Reponses: Number +	Number ±	Number −	Percent total +	Percent total + and ±
MZ	neg[b]	0	1	43	0	2.3
KLSVY	neg	2	16	92	1.8	16.3
P	trace[c]	1	3	18	4.5	18.2
R	trace[c]	1	7	14	4.5	36.4
U	trace[c]	2	7	13	9.1	40.9
A	0.0015	0	1	21	0	4.5
B	0.0020	3	4	15	13.6	31.8
C	0.0040	4	6	12	18.2	45.4
X	0.0040	0	15	7	0	68.2
O	0.0052	10	9	3	45.4	86.3
Q	0.0058	9	12	1	40.9	95.4
J	0.0070	19	3	0	86.4	100.00
W	0.0084	20	2	0	90.9	100.00
N	0.0086	21	1	0	95.4	100.00
T	0.0086	14	7	1	63.6	95.4

[a]Samples A, B, C, and J, assayed by *B. stearothermophilus* var. *calidolactis* method (Gist-Brocades); others by *S. lutea* (FDA). These were assayed on a dry basis and the final concentrations obtained by calculation.

[b]No zone with *S. lutea;* probably less than 0.002 unit/ml.

[c]Zone with *S. lutea* but not assayable; probably between 0.002 and 0.004 unit/ml.

Samples other than A, B, C, and J of Table 2 were assayed by FDA using the *S. lutea* cylinder cup method on 1/4 dilutions of milk powder. The *S. lutea* method was not sensitive enough for A, B, C, or J samples. These, however, were assayed by Gist-Brocades using a cylinder cup method and spores of *B. stearothermophilus* var. *calidolactis.* The other samples were also assayed by Gist-Brocades, with the following results expressed as 1/11 water dilutions of the powders in units/ml penicillin: samples K (0.0028), L (0.0021), N (0.0056), O (0.0052), P (0.0030), Q (0.0050), R (0.0030), S (0.0020), T (0.0035), U (0.0021), V (0.0012), W (0.0053), X (0.0023), and Y (0.0013). The FDA *S. lutea* method (Table 2) showed no zone in K, L, S, V, or Y and a small zone beyond the range of the test for P, R, and U. The greatest discrepancy was in sample T with the FDA method showing more than twice the pencillin concentration of the Gist-Brocades test.

Regression lines of type of response on penicillin concentration

Prediction equations in the range of about 0.001 to 0.01 unit/ml for determining the probablility of obtaining a predetermined percentage of + or + and ± responses from various penicillin levels were derived using the data from Table 2 (from FDA and Gist-Brocades). The correlation coefficient (r) for both + and + or ± responses was 0.91 with 95% confidence limits of 0.82 to 0.95. The regression equation from the FDA data for determining penicillin concentration (X) needed to give a predetermined probability of desired + responses (Y_1) was

$$Y_1 = -25.2 + 12823.9\ X$$

and the equation for + and ± responses (Y_2) was

$$Y_2 = 7.69 + 11803.5\ X$$

By setting $Y_1 = 0.95$ (95% probability of obtaining only + responses), the corresponding penicillin level would need to be 0.0094 unit/ml; the same probability for Y_2 would require a penicillin concentration of 0.0074 unit/ml. Likewise for $Y_1 = 1.00$, X would be 0.0098 unit/ml, and for $Y_2 = 1.00$, X would be 0.0078 unit/ml.

Similar equations using the more sensitive methods of Gist-Brocades for determining pencillin levels gave a 95% probability value for Y_1 of 0.0072 unit/ml (the equation was $Y_1 = 2.943 + 17406.4\ X$, with $r = 0.87$ and confidence limits 0.67 and 0.95) and a value for Y_2 of 0.0056 unit/ml (the equation was $Y_2 = -1.55 + 17374.6\ X$, with $r = 0.81$ and confidence limits of 0.55 and 0.92).

Other antibiotics

In the 22 tests conducted, the three concentrations of tetracycline, 0.192, 0.089, and 0.058 mcg/ml, respectively gave + responses of 16, 0, 0; ± responses of 2, 2, o; and − responses of 4, 20, 22. The neomycin concentrations of 26.5, 19.0 and 9.0 mcg/ml, respectively, gave + responses of 20, 14, and 8; ± responses of 0, 7, 13; and − responses of 2, 1, 1.

DISCUSSION

There are two areas in which antibiotic residue testing is important in milk. One is for incoming bulk tank raw milk where a simple, rapid, and sensitive test is necessary; the other is for dry milk where the method need not be as rapid but should still be simple and sensitive. The *S. lutea* test does not meet these criteria; it requires skilled personnel, is laborious and time-consuming, and requires considerable laboratory equipment. The *B. subtilis* disc assay, especially the rapid modification (*20*), is fairly simple but less sensitive than other tests. The *B. stearothermophilus* and *B. stearothermophilus* var. *calidolactis* tests appear better suited for qualitative field testing of milk. The Delvotest P modification (other variations could easily be developed by interested laboratories) is particularly simple, rapid, and sensitive.

The relative claimed sensitivities of these methods are 0.01 unit/ml penicillin for the *S. lutea* method (*11*); 0.05 for the *B. subtilis* disc method (*19, 20*); and 0.004 for the *B. stearothermophilus* var. *calidolactis* disc method as

reported by Forschner (*4*) and Van Os et al. (*17*). The latter workers claimed that samples with a concentration of 0.004 unit/ml gave 100% positive results. Similar results were obtained by one of us (CNH), probably because of familiarity with the technique. The study reported here was undertaken by analysts who were unfamiliar with the method; even they, however, reported a large proportion (83%) of + or ± responses (29% +). The concentration of penicillin which would produce + results 95% of the time (0.0072 and 0.0094 unit/ml from the regression equations using FDA data) indicated that the sensitivity of the Delvotest P method is greater than that of the *B. subtilis* disc method and approximates that of the *S. lutea* method. Reconstitution of dry milk powders to 1/4 instead of 1/11 would increase sensitivity.

Van Os et al. (*17*) indicated that the *B. stearothermophilus* var. *calidolactis* method could detect 0.40 mcg tetracycline/ml of milk. Our results on 22 tests, showing 16 + responses at the 0.192 mcg/ml level, support this estimate of sensitivity. Van Os et al. also indicated that neomycin gave 100% + responses at 22 mcg/ml. Our results were similar: 20 + responses of 22 tests at 26.5 mcg/ml. Lower concentrations under our conditions gave more ± rather than − responses. This type of response, also noted during the initial preparation of the neomycin powders in the coordinating laboratory, is probably due to slower diffusion but may also be due to pH effects as indicated by van Os et al. (*17*).

A problem with the Delvotest P method is interpretation of the ± reaction. Our results tend to indicate that the ± reaction should be considered +, since a large proportion of the ± responses became + in tests of the more concentrated 1/4 dilution. However, it is not practical to concentrate fluid milk under ordinary conditions. Laboratories experiencing uncertainty over the interpretation of the ± reactions in raw or dried milk dilutions could resolve this in another way by further diluting by 1/2 or 1/4; the reaction should then revert to −. If the reaction after dilution is still ±, then low levels of neomycin or other antibiotics might be present. The penicillinase test would verify presence of penicillin.

The present practice of assaying dry milk powders at a 1/4 dilution will give greater sensitivity as we demonstrated; however, for enforcement purposes, this practice is of doubtful value since the same sensitivity could not be attained in the parent raw milk. Dry milk should be assayed at 1/11 dilution, approximating that of raw milk.

Adoption of a method such as the Delvotest P modification of *B. stearothermophilus* var. *calidolactis* test should be considered only after all ramifications are considered. For instance, the greater sensitivity would undoubtedly mean that more milk would be condemned. What would be the impact on the producer or the distributor? The cost of this test would also be considerable (at present about 56 cents per test) if used on all farm bulk tanks. Perhaps this test should be used only for tank car lots of commingled milk where the greater sensitivity would be an advantage; while the less sensitive, cheaper, equally-as-rapid *B. subtilis* disc method could be used, as it now is, for farm bulk tanks. The *S. lutea* test appears to have no advantages over the *B. stearothermophilus* var. *calidolactis* test and has several disadvantages including extra time required, unstable bacterial culture, and the necessity for trained personnel. The ideal solution would be a disc method with *B. stearothermophilus,* similar to the *B. subtilis* method. This is now under consideration and is being studied in several laboratories.

CONCLUSIONS

The *Bacillus stearothermophilus* var. *calidolactis* test (Delvotest P) is a good field technique for detecting antibiotics in raw milk or dry milk powder. Regression equations indicated that milk containing about 0.008 to 0.010 unit of penicillin/ml would be detected 95% of the time by untrained analysts.

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