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USE OF MARKERS IN VETERINARY PREPARATIONS FOR THE DETECTION OF ANTIBIOTICS IN MILK

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The frequent occurrence of veterinary antibiotics in the present-day milk supply has been the concern of a number of individuals in many fields of endeavor. In the past few years, there appears to have been an increasing interest on the part of regulatory officials, as well as industry personnel, in the incidence of antibiotics in milk (7, 8). The public health significance of antibiotic residues in market milk and its regulatory aspects have been thoroughly covered in the preceding paper by Grove (2) and certainly need no re-emphasis.

The Agricultural Research Service of the U. S. Department of Agriculture has a twofold interest in this problem. It is fully aware of the gross economic losses to the dairy farmer from mastitis and it appreciates the need for antibiotics to control this disease. At the same time, the Department is equally disturbed because such antibiotics can and occasionally do occur in sufficient concentration to cause serious economic losses to the cheese and fermented milk industries. That veterinary antibiotics are capable of inhibiting dairy starter microorganisms is well documented.

Hunter (6) in 1949 was among the first to point out the effects of penicillin on Cheddar cheese starters. Hargrove *et al.* (5) in 1950 reported on the effects of penicillin and streptomycin on Swiss cheese starters. Several sensitive and apparently reliable tests for antibiotics in milk have been proposed recently, all of them laboratory tests that require from 2.5 to 8 hr. to run. The time, laboratory equipment, and skills required reduce their usefulness, particularly to cheese-makers and dairy farmers. It appears evident that a microbial assay for antibiotics would not be practical from the standpoint of testing many milks as they are received.

A sizable list could be made of the various proposals that have been offered as a means of controlling antibiotics in the market milk supply. Many of the proposals that would be satisfactory from a public health standpoint would be far from beneficial to the milk producer or manufacturer of cheese and fermented milks. Some would have penicillin banned from all

intramammary veterinary preparations; others would ban all the antibiotics used in human medicine. As far as is known, all of the veterinary antibiotics are capable of inhibiting dairy starter microorganisms and, therefore, the substitution of one antibiotic for another would be of no assistance to the dairy manufacturers. Some workers in the field of veterinary science have advocated that the treatment of mastitis with antibiotics be restricted to veterinarians. Although this might be an ideal situation, it does not guarantee that milk containing antibiotics will not enter into the general milk supply. Undoubtedly, the educational programs that have been sponsored by state and local groups have had some beneficial effect. However, our investigations into actual cases and causes of cheese starter failures would indicate that the problem is still with us.

It appears that a satisfactory solution to the problem of antibiotic residues should and must consider the viewpoints and needs of the milk producer and manufacturer, in addition to meeting the requirements from a health standpoint.

If the dairy farmer could be provided with a practical means whereby he could detect the antibiotic as it appears in the milk of a treated animal, he would be less apt to forget and include such milk in his supply. With a rapid and reliable means of detection, the manufacturer could detect antibiotic-containing milks as they were received at the plant and, thereby, divert such milks from his operation. Before this laboratory initiated its present tracer-dye studies, many cheese and dairy manufacturers indicated their urgent need for a rapid means of detecting antibiotics in the producer's milk as it is received. Therefore, the present study was designed to explore the feasibility of adding dyes to veterinary antibiotics as a means of indirectly detecting antibiotics in milk.

In 1950, we entertained the idea of incorporating an innocuous certified food color in veterinary preparations. Our interest at the time was prompted by a few starter failures in our experimental cheese work. The work was not pursued when it was found that the dye,

amaranth red, was not excreted in the milk as long as was penicillin. It was apparent, then, that an ideal dye should be detectable in the milk from a treated quarter for as long a time as the antibiotic. Naturally, it could not be toxic to the cow, affect the level of milk production, or degrade the antibiotic activity of the drug. Very little interest was noted in such a proposal at that time. Since then, several workers have advocated the use of dyes or coloring agents to color the milk following treatment of dairy cattle with antibiotics. However, very little has actually been published on the subject.

In 1956, this laboratory participated in a conference sponsored by the Food and Drug Administration on "Antibiotic Residues in Market Milk." The fluorescent-marker study was in its early stages at the time but, as it showed some promise, it was suggested as a means of dealing with the problem. However, the Medical Advisory Panel at the time did not seem very receptive to the idea of a marker in veterinary preparations. Dalgaard-Mikkelsen and Rasmussen (1), 1957, in Denmark, have published their preliminary tracer-dye study, in which they used a green dye called "Green S." They indicated that the dye is very effective in coloring the milk after treatment, and suggested that its addition would be a reliable means of controlling antibiotics in milk.

Guthrie and Kosikowski, at Cornell University, have also been studying the possibility of including dyes in mastitis preparations. Their work was described by Guthrie (3) at the 1957 New York State Veterinary Convention. At the time, he stated that oil-soluble chlorophyll showed more promise than any of the materials they had tested. The preliminary report of Hargrove *et al.* (4), on the use of fluorescent materials for the detection of antibiotics in milk, has appeared recently.

The tracer-dye work to be reported here, for the most part, is a continuation of that study. The work has been undertaken in a joint project between the Dairy Laboratories of EURDD and AHRD of the Agricultural Research Service, USDA, in cooperation with the Division of Antibiotics of the Food and Drug Administration.

As previously indicated, this laboratory has been primarily interested in testing fluorescing compounds as markers, because fluorescent materials are known to be easily detected in trace amounts with ultraviolet light. The preliminary marker study was divided into the following phases: (1) The screening of a number of possible marker materials in milk; (2) storage studies of marker mixed with penicillin preparations; (3) the administration of marker-penicillin preparations to a representative number of cows; (4) detecting the effect of marker on the cows, and (5) analysis and correlation of dye excretion with penicillin excretion in milk.

Having selected a marker on the basis of the

preliminary work, the study was extended to include: (1) The development of a quantitative test for the marker; (2) a study of the effect of markers on ten different commercial antibiotic preparations throughout storage; (3) more intensive study of the effect of the marker on different cows; (4) a comparison of the rate of marker excretion in eight different commercial veterinary preparations, and (5) the treatment of actual cases of mastitis with different veterinary products containing marker.

Screening studies. The list of chemicals screened for marker properties included certified food colors, cosmetic dyes, food-flavoring compounds, and a number of fluorescing materials such as the fluoresceins, chlorophylls, and hydroxycoumarins. The fluorescent materials were added to milks and these were diluted serially and measured for fluorescence with an inexpensive 2-amp., long-wave ultraviolet lamp. The fluorescent materials as a whole were superior to other compounds, with respect to ease and limit of detection in whole milk. Methyl-anthranilate, esculin, 4-methyl umbelliferone, the chlorophylls, and the fluoresceins were selected for further study.

Storage studies. None of these materials caused a loss of penicillin activity when mixed with penicillin in oil and stored for a period of 4 mo. Some loss of penicillin activity was noted when penicillin and chlorophyll were stored in the vehicle Peniele.

Udder infusions. Three separate trials were conducted to evaluate the marker materials by udder infusion. Some markers were subjected to additional trials if further testing seemed advisable. Each trial was designed as a balanced incomplete block experiment. Four marker treatments were assigned at random to the four quarters of the udder of each of six cows representing a wide range in milk production.

Quarter-milkers were used to collect samples of the milk from the individual quarters every 12 hr., for at least 96 hr. after infusion. Close veterinary scrutiny and milk production records were maintained. Milk samples from the treated quarters were tested for leucocytes, pH, antibiotic content, and presence of marker. Milks containing marker were diluted with normal herd milk to determine the extent of dilution possible without loss of marker detectability.

Marker effect on cows. 4-Methyl umbelliferone was irritating to the udders of the test animals, and the milk from the treated quarters was definitely abnormal. Much of the methyl anthranilate appeared in the urine of the treated animals. No evidence of toxicity was noted in the udders of the cows treated with oil-fluorescein and uranine (sodium fluorescein).

Correlation studies. A combination of 125 mg. of oil-soluble fluorescein and 125 mg. of uranine per dose of antibiotic showed greater correlation with the excretion of penicillin than did the other markers. It could be detected visually in the milk for 48 hr. after infusion and up to 96 hr. with ultraviolet light. The

marker colored both the fat and nonfat portion of the milk. Its presence should preclude the use of any portion of the antibiotic-containing milks for manufacturing purposes. The cost of the fluorescein markers material should not amount to more than 1/2¢ per dose.

Figure 1 presents representative data which show a close correlation between the excretion of penicillin and the fluorescein marker. The dye in these cases persisted in the udders of three cows (No. 2466, 3443, and 3457), just about the same length of time as the antibiotic. It was observed that the level of milk production markedly affected the excretion both of marker and of antibiotic from the quarter. In this study, the level of milk production of the

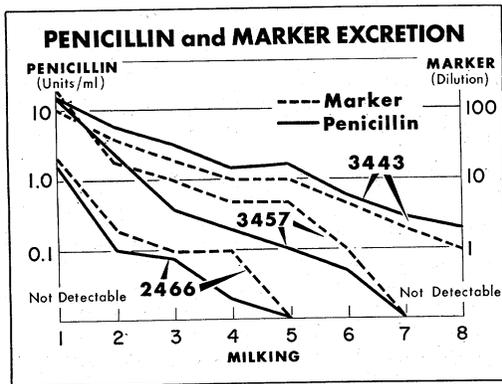


FIG. 1. Correlation between the excretion of penicillin and marker.

cows ranged from 10 to 50 lb. per day. As might be expected, penicillin and marker persisted anywhere from 24 to 48 hr., or longer, in the lower producers. The two upper curves show the rate of excretion in a cow producing about 30 lb. of milk, and the two lower curves are from a cow giving over 40 lb. per day. The two center curves are for a cow producing between 30 and 40 lb. Although individual differences in cows, and other factors, appeared to influence the rate of marker and antibiotic excretion, the level of milk production had the most dramatic effect.

Figure 2 shows the typical rate of excretion of marker and antibiotic from a cow giving about 35 lb. of milk. The cow was injected with 15 ml. of a marker-penicillin preparation. The dye could not be detected visually in the milk after the fourth milking. With the ultraviolet lamp, the dye was detected up to the seventh milking. Assays for penicillin were negative after the seventh milking. It was observed that the milks containing about ten units of penicillin per milliliter could be diluted 100 times and still have the marker detectable by the ultraviolet light.

Analysis for marker. The procedure used for the detection of marker by the ultraviolet lamp, although very effective, was at best only semi-

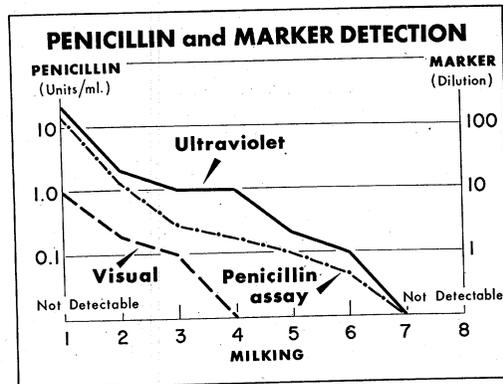


FIG. 2. Rate of excretion of penicillin and marker.

quantitative. Determinations of the actual dye content of the milk seemed necessary to provide quantitative data. Therefore, quantitative procedures were developed for measuring the oil-fluorescein and uranine separately. Briefly, the procedure for oil-fluorescein consisted of acidifying the sample with sulfuric acid, adding sodium tungstate, and extracting with ethyl ether. Color was measured in a Model B spectrophotometer at a wave length of 420 m μ . A standard curve was used to compute the micrograms of marker in the sample. The determinations for uranine also were made by a spectrophotometric procedure. In brief, the sample was treated with *N* NaOH, filtered, and measured for color at a wave length of 480 m μ .

Degree of marker toxicity. Two different methods were followed in an attempt to determine if increased doses of marker would have a toxic effect on the cows. In one instance, the marker was added to the veterinary preparation at the rate of 250 mg. of oil-fluorescein and 125 mg. of uranine per dose. Three cows were treated with this preparation for four successive milkings. No evidence of a toxic reaction could be detected. In a second procedure, a massive dose of the marker was injected into the udder of six cows. No evidence of toxicity was noted with eight times the prescribed dose in two cows. Increased dosages up to 16 times the normal, or 2 g. per dose, caused no effect on two cows. However, two out of four cows evidenced a slight swelling in the quarter on the third day after injection.

Commercial antibiotic storage study. The effect of the marker on the antibiotic activity of various commercial antibiotics during storage is being studied. Ten different commercial veterinary preparations were selected for the storage study. The antibiotics represented in the preparations were penicillin, dihydrostreptomycin, bacitracin, neomycin, polymyxin, erythromycin, chlortetracycline, and streptomycin. One or more sulfonamides also were in several preparations. Among the vehicles in the preparations were mineral oil, sesame oil, peanut

oil, and lanolin. The marker was added to each preparation at the rate of 250 mg. of oil-fluorescein and 125 mg. of uranine per dose. The mixtures were stored at room temperature. Assays for the respective antibiotics were performed at monthly intervals for 7 mo. No appreciable loss of antibiotic activity was noted in seven of the preparations; whereas, in three there was a marked decrease in the activity of one or more components. Thus, neomycin was stable in two products, unstable in one; penicillin was stable in six, unstable in two; polymyxin was stable in one, unstable in one; bacitracin, stable in one; chlortetraacycline, stable in one; dihydrostreptomycin, stable in four; erythromycin, stable in one, and streptomycin, stable in one. These product-to-product differences in stability suggest something more than antagonism between marker and antibiotic.

Persistence of marker in udder. It seemed that the rate of marker excretion and persistency in the udder might vary considerably among the numerous commercial veterinary preparations on the market. To explore this possibility, eight commercial preparations were selected to be tested by udder infusion with marker. The preparations represented most of the common vehicles, and the prescribed dosages on the labels ranged from 6 to 28 ml. The marker was added to the preparations at the rate of 250 mg. of oil-fluorescein and 125 mg. of uranine per dose. Four cows were injected with the eight preparations in two different trials. The test was designed so that each quarter of an udder in two periods received all eight treatments, with no quarter receiving the same treatment in both periods. Data from these trials were analyzed statistically. An analysis of variance in the number of milkings required to reach a certain dilution showed that there was no marked difference between the type of commercial preparation and the rate of marker excretion. Figure 3 shows the range of marker excretion of the eight products in four cows. The values for each preparation fell within the range that is indicated by the shaded area. The

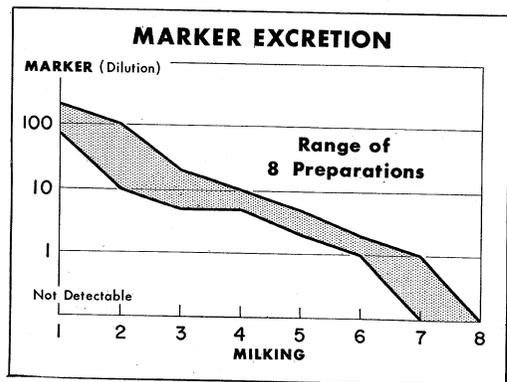


FIG. 3. Range of marker excretion from commercial veterinary preparations.

marker in each preparation disappeared by the seventh and eighth milking.

Mastitis treatment. In all, 18 cases of mastitis have been treated with nine different commercial antibiotics to which the fluorescein marker was added. Milk samples were collected for 96 hr. after treatment, measured for marker content, and assayed for each of the antibiotics that were in the original preparation. The values obtained were used to correlate the persistence of marker and antibiotics in the udder. Figure 4 represents a mastitis

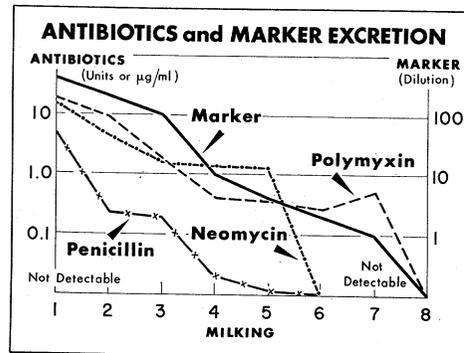


FIG. 4. Excretion of multiple antibiotic and marker from a mastitis quarter.

case that was treated with a multiple antibiotic that contained marker. The marker remained in the quarter as long as the most persistent antibiotic (polymyxin).

In conclusion, it may be re-emphasized that the first objective of the marker-dye study was to explore the possibility of such a procedure and, secondly, on the basis of favorable results, it seemed necessary to obtain sufficient data for evaluation and presentation to the proper authorities for a decision or ruling. The oil and water-soluble fluorescein-marker was definitely superior to all of the materials that we have tested. It is believed that its addition to veterinary antibiotics would be of considerable value in eliminating antibiotic residues from the general milk supply.

However, since this work was completed, some doubt has been cast by the Food and Drug Administration on whether the addition of "inactive" (nontherapeutic) ingredients, such as these dyes, to mastitis preparations would be advisable, since it might possibly result in residues of the dyes in the milk supply.

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