Inactivation of Penicillin G in Milk Using Hydrogen Peroxide

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ABSTRACT

Milk antibiotic residues have been a public concern in recent years. The Grade A Pasteurized Milk Ordinance mandates that raw Grade A milk will test negative for β -lactam antibiotic residues before processing. The purpose of this research was to investigate the ability of various levels of peroxide and heat to inactivate penicillin G in raw milk. Whole milk spiked to a mean of 436 ± 15.1 (standard error of the mean) ppb of potassium penicillin G was treated with hydrogen peroxide at levels of 0.0, 0.09, 0.17, and 0.34%. Samples at each peroxide level ($n = 6$ per treatment) were treated as follows: 1) incubated at 54.4°C for 3 h, 2) pasteurized at 62.8°C for 30 min, 3) incubated and pasteurized as in treatments 1 and 2, or 4) received no further treatment. A β -lactam competitive microbial receptor assay was used for quantification of penicillin G. Concentrations of penicillin in selected samples were determined by HPLC for a comparison of test methods. Treatments were evaluated relative to their ability to reduce milk penicillin G levels to below the safe level of 5 ppb.

The 0.09% hydrogen peroxide level was ineffective for all treatments. Hydrogen peroxide at 0.17% lowered the mean penicillin G ($\pm$ SEM) from 436 ± 15.1 to 6 ± 1.49 ppb using the incubated and pasteurized heat treatment. The 0.34% concentration of hydrogen peroxide was the most effective, inactivating penicillin G to a level well below the safe level of 5 ppb with the pasteurized heat treatment, with or without incubation.

(Key words: milk, penicillin, antibiotic, hydrogen peroxide)

INTRODUCTION

Penicillin G is commonly used to treat mastitis and other infection disorders of cattle. Penicillin may be problematic in the milk supply due to the possibility of allergic reactions (Sullivan et al., 1981) and the poten-

tial spread of antibiotic resistance (Rice and Carias, 1998). However, at low levels, this has not been documented. Penicillin restricts the growth of lactic acid bacteria in milk, which is a problem for cheese makers because it affects the starter culture (Hunter et al., 1949; Hansen et al., 1950). Yogurt starter cultures are more susceptible to penicillin than cheese starter cultures (Cogan, 1972).

The thermal stability of antibiotic structures and properties is not widely reported in the literature. Inactivation of antibiotics in milk, water, and buffer solutions has been reported (Shahani et al., 1956; Konecny, 1978; Moats, 1999). One report showed a 30.0% reduction of penicillin when milk containing penicillin was heated at 71°C for 30 min and stored for 7 d (Shahani et al., 1956). Pilet et al. (1969), as cited by Moats (1999), demonstrated a reduction of 85 to 100% when penicillin G at concentrations of 0.15 to $0.25 \mu\text{g/mL}$ was heated for 90 min at 100°C .

The earlier tests for determining antibiotic residues used agar and examined the inhibition of bacterial growth on a plate. These tests were sensitive, but antibiotics could not be specifically identified (Moats, 1997). A variety of tests have been developed to detect antibiotic residues in milk. Among these, a competitive microbial assay, the Charm β -lactam test, is very sensitive and does not use bacterial growth as part of the methodology (Charm Sciences, Inc., 1997).

Hydrogen peroxide has been used as a preservative in milk in developing countries. It is reported to be an "excellent and safe preservative" in areas where there is no refrigeration (Williams, 1966). The US FDA approved hydrogen peroxide for treating milk used in Cheddar and Swiss cheese manufacturing in 1962 (Wendorff, 1990). The weight of hydrogen peroxide cannot exceed 0.05% of the milk weight (Wendorff, 1990). Hydrogen peroxide has been used with riboflavin plus lactoperoxidase to inactivate aflatoxin in contaminated raw whole milk (Applebaum and Marth, 1982). Hydrogen peroxide and potassium sorbate were used to retard the growth of psychrophilic bacteria in milk (Mistry and Kosikowski, 1985).

There are potential concerns with use of hydrogen peroxide in milk. Problems have occurred when farmers added hydrogen peroxide to bulk tank milk with high

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bacterial counts (Wendorff, 1990). Concentrations as low as 5 ppm will inhibit lactic acid bacteria that are used to produce cultured products. Hydrogen peroxide at a 0.01% concentration resulted in increased casein proteolysis with rennin and this led to softness in the cheese (Wendorff, 1990). Flavor may be affected by a 0.01% peroxide concentration, with bitter and acid flavors after aging (Fox and Kosikowski, 1967).

There have been limited studies using hydrogen peroxide to inactivate β -lactam antibiotics in milk. Destruction of penicillin was observed when 1.05% hydrogen peroxide was added to milk containing 1 U/mL of penicillin and incubated at 45°C (Fox, 1965). Hydrogen peroxide at levels of 0.03 to 0.16% with heating at 63°C for 11.5 h inactivated penicillin at concentrations of 0.04 and 0.1 U/mL, respectively (Simetskii, 1973).

One study documented the formation of aminoxyl radicals when penicillin was degraded using hydrogen peroxide in water solutions of pH 7 to 8 (Lagercrantz, 1992). The reaction involved the oxidative cleavage of the β -lactam ring of penicillin with the formation of a cyclic aminoxyl radical, in which the thiazolidine ring carried the nitroxide group (Lagercrantz, 1992).

The purpose of this study was to determine the ability of various combinations of hydrogen peroxide and/or heat treatments to reduce penicillin G in milk from initial levels of 436 ± 15 ppb penicillin G to levels below the US FDA safe level of 5 ppb.

MATERIALS AND METHODS

Design

The experiment considered 4 levels of hydrogen peroxide (0.0, 0.09, 0.17, and 0.34%) and 4 heat treatments (control at 1.1°C, incubation at 54.4°C for 3 h, pasteurization at 60.6°C for 30 min, or incubation [as above] followed by pasteurization [as above]). There were 6 tubes in each peroxide-heat treatment combination, with 3 replications.

Milk Source and Spiking Milk with Penicillin

Raw skim milk from the North Carolina State University dairy plant was spiked with potassium penicillin G (Sigma Chemical Co., St. Louis MO) to a mean ($\pm$ SEM) level of 436 ± 15.09 ppb. Hydrogen peroxide (Fisher Scientific Co., Fairlawn, NJ) was added to milk at 0.0, 0.09, 0.17, and 0.34% ($n = 6$) (no. 1367 milk dilution bottles, Corning Glass Co., Corning, NY) and shaken (junior orbit shaker, Lab-Line Instruments, Inc., Melrose Park, IL) at 150 rpm for 15 min. While shaking, the control was kept at 1.1°C and the other 3 treatments were kept at 27°C. To clean them before the experiment started, the milk bottles were soaked

in a hot water bath with 10 mL of sulfuric acid added (Fisher Scientific Co.), rinsed 3 times with deionized water, and dried in a drying oven (Stabil-Therm drying oven, Blue M Co., Blue Island, IL).

Heat Treatments

After shaking, all levels of penicillin other than control were incubated at 54.4°C for 3 h in an incubator (model LEB-1 to 75, Despatch Co., Minneapolis, MN), pasteurized at 60.6°C for 30 min in a water bath (model 16-X-2, Precision Scientific Co., Chicago, IL), or incubated and pasteurized.

Analysis of Drug Concentrations

Concentrations of penicillin G in milk were determined using a microbial receptor assay (Charm II Beta-Lactam, Charm Sciences Inc., Malden, MA), which used bacteria with specific receptor sites that bind to all β -lactam drugs. The bacteria were added to a milk sample along with 0.15 kilobecquerels of ^{14}C -labeled penicillin G. Any β -lactam analog already in the milk competed for the binding sites with this labeled penicillin G. The amount of ^{14}C -labeled penicillin G that bound to the receptor sites was measured and compared with a previously determined control point or evaluated using a standard curve. The greater the amount of ^{14}C present and measured by the scintillation counter, the lower the β -lactam concentration in the sample (Lagercrantz, 1992).

The Charm II test for β -lactam antibiotics was used according to the manufacturer's directions for quantitative testing (Charm Sciences, Inc., 1990, 1992). A standard curve was made using known concentrations of penicillin G (0, 1.25, 2.5, 5.0, and 10 ppb), with 6 replicates for each concentration. The counts per minute, B from the sample, was divided by the B0 counts per minute, determined from the zero count (raw skim milk containing no penicillin G) used in this experiment. This fraction, called B/B0, was used on the y-axis. The penicillin concentration associated with each value was read off the x-axis from the standard curve.

HPLC Analysis

Samples that had already been tested using the Charm method were sent "blind" to an independent testing laboratory (Silliker Laboratories, Chicago Heights, IL) for HPLC analysis of penicillin G using the method of Moats (1990). All samples with Charm-determined values below 10 ppb from the number 2 replications from each treatment level were evaluated.

Table 1. Least squares means¹ natural logarithms ppb penicillin G by peroxide level and incubation and pasteurization treatments (back-transformed values in parentheses)

Peroxide level (%)	Incubation	Not pasteurized	Pasteurized
0.0	No	6.03 (436) ^{wy}	6.04 (428) ^{wy}
0.0	Yes	5.95 (377) ^{wy}	5.93 (382) ^{wy}
0.09	No	6.06 (415) ^{wy}	4.63 (102) ^{wz}
0.09	Yes	4.94 (153) ^{xy}	4.31 (83.3) ^{xz}
0.17	No	5.40 (227) ^{wy}	2.17 (12) ^{wz}
0.17	Yes	3.40 (43) ^{wy}	1.80 (6) ^{xz}
0.34	No	4.85 (135) ^{wy}	0.85 (1.4) ^{wz}
0.34	Yes	1.52 (8) ^{xy}	0.80 (1.4) ^{wz}

¹Standard errors, 0.09 to 0.11.

^{w,x}Within peroxide level, values within columns with different letters are significantly different, $P < 0.05$.

^{y,z}Within peroxide level, values within rows with different letters are significantly different, $P < 0.05$.

Hydrogen Peroxide Test

Test strips that detect hydrogen peroxide levels above 1 ppm (Serim peroxide reagent test, Elkhart, IN) were used to detect hydrogen peroxide in milk (Serim Research Co., 1996). After 15 s, hydrogen peroxide was quantitated (0 to 10 ppm) based on detection of a color change.

Statistical Analysis

Data were analyzed as natural log transformations of values of penicillin G ppb plus one [$\ln(\times \text{ppb penicillin G} + 1)$]. The experimental design was a $4 \times 2 \times 2 \times 2$ factorial with 3 replications or trials, considering the main effects of trial, peroxide level, incubation, pasteurization, and appropriate interactions. Statistical significance was considered present at $P < 0.05$.

RESULTS AND DISCUSSION

Least square mean $\pm$ SEM penicillin G concentrations in milk of peroxide and heat treatments are shown in Table 1. Analysis indicated highly significant ($P < 0.001$) differences among peroxide levels with marked linear effect. Significant ($P < 0.001$) peroxide level $\times$ incubation, peroxide level $\times$ pasteurization, and peroxide level $\times$ incubation $\times$ pasteurization interactions were observed.

With no heat treatment, the 0.0% peroxide (control) level had a mean $\pm$ SEM of 436 ± 15.1 ppb penicillin G for the 3 trials. For the incubation treatment with 0.0% level of peroxide, the mean $\pm$ SEM penicillin G concentration was reduced to 377 ± 19.1 ppb. For the pasteurization treatment at the 0.0% level of peroxide, the mean $\pm$ SEM level was 428 ± 15.3 ppb. For the incubation and pasteurization treatment, the mean $\pm$ SEM penicillin G level was reduced to 382 ± 21.0 ppb.

Table 2. High-performance liquid chromatography vs. Charm test Penicillin G concentration for samples with Charm-determined values < 10 ppb

Sample #	% H ₂ O ₂	Treatment	HPLC	Charm
322	0.17	Incubation (Inc)	4.3	4.6
332	0.17	Pasteurization (Past)	3.6	4.8
342	0.17	Inc and Past	3.2	1.4
422	0.34	Incubation	2.9	1.9
431	0.34	Pasteurization	2.5	0.5
432	0.34	Pasteurization	2.7	1.8
441	0.34	Inc and Past	2.8	1.4
442	0.34	Inc and Past	3.8	1.8

The 0.09% hydrogen peroxide is not as effective in decreasing the level of penicillin G below the safe level of 5 ppb, even when using the heat treatments. With no heat treatment, the mean $\pm$ SEM level of penicillin was 415 ± 14.5 ppb. With incubation, the mean $\pm$ SEM level of penicillin was 153 ± 12.1 ppb. With pasteurization, the mean $\pm$ SEM level of penicillin was 102 ± 10.54 ppb. With incubation and pasteurization, the mean $\pm$ SEM level of penicillin was 83 ± 8.6 ppb.

Using a 0.17% level of hydrogen peroxide with no heat, the mean $\pm$ SEM level of penicillin dropped to 227 ± 14.9 ppb. Incubation alone lowered the mean $\pm$ SEM level of penicillin to 43 ± 8.5 ppb. With pasteurization, 0.17% peroxide lowered the mean $\pm$ SEM level of penicillin to 12 ± 2.3 ppb. Incubation and pasteurization reduced the concentrations of penicillin to a mean $\pm$ SEM level of 6 ± 1.5 ppb, quite close to the safe level.

The 0.34% level of hydrogen peroxide lowered the mean $\pm$ SEM level of penicillin G to 135 ± 15.2 ppb with no heat and to 8 ± 3.4 ppb when incubated. Pasteurization dropped the mean $\pm$ SEM level to 1.4 ± 0.2 ppb. When both incubated and pasteurized with 0.34% hydrogen peroxide, penicillin G was reduced to a mean $\pm$ SEM level of 1.4 ± 0.2 ppb, well below the safe level of 5 ppb.

The HPLC and Charm test results are shown in Table 2 for microbial receptor assay-determined values less than 10 ppb analyzed blindly by HPLC. The comparison indicates good agreement between HPLC- and Charm-determined concentrations of penicillin G for these samples.

Incubation at 54.4°C for 3 h was used to determine if enzymes already present in the milk (peroxidase, hydroperoxidase, lactoperoxidase, xanthine oxidase, catalase) would break down the residual peroxide in milk. All samples tested were positive for peroxide, indicating that this time-consuming step did not reduce the level of residual peroxide.

These experiments showed that hydrogen peroxide at a level of 0.34% with pasteurization and/or incubation reduced penicillin G to below the safe level as detected

by the microbial receptor assay. Both pasteurization and incubation with pasteurization were similar in effect. The 0.17% peroxide level, using incubation and pasteurization, reduced the mean $\pm$ SEM level of penicillin G to 6 ± 1.5 ppb, very close to the "safe" level of 5 ppb. These results indicate that peroxide with incubation and/or pasteurization can be used to inactivate penicillin G in milk.

CONCLUSIONS

Milk contaminated with penicillin G at 436 ± 15.1 ppb was treated with 0.0, 0.09, 0.17, and 0.34% hydrogen peroxide. Samples at each peroxide level ($n = 6$ per treatment) were treated as follows: 1) incubated at 54.4°C for 3 h; 2) pasteurized at 62.8°C for 30 min; 3) incubated and pasteurized as in treatments 1 and 2 or 4) received no further treatment. A β -lactam competitive microbial receptor assay was used for quantification of penicillin G. Concentrations of penicillin in selected samples were determined by HPLC for a comparison of test methods. Hydrogen peroxide at 0.17% lowered the mean penicillin G ($\pm$ SEM) from 436 ± 15.1 to 6 ± 1.49 ppb using the incubated and pasteurized heat treatment. The 0.34% concentration of hydrogen peroxide was most effective, inactivating penicillin G to a level well below the safe level of 5 ppb with the pasteurized heat treatment, with or without incubation.

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