Thermal Destruction of Staphylococcus aureus in Human Milk

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ABSTRACT

Rates of thermal destruction of Staphylococcus aureus were determined in mature human milk using a continuous flow high-temperature short-time pasteurization system. D and z values for inactivation of S. aureus were determined from data on survivors capable of forming colonies in an appropriate selective medium. The effects of thermal injury on D and z values were assessed by survivor colony forming units (CFU) on Staphylococcus medium 110 (SM110), nutrient agar (NA), Trypticase Soy Agar (TSA), Trypticase Soy Agar with 7.5% NaCl (TSAS) and Baird-Parker medium (BP). D-values for inactivation of S. aureus at 52, 58 and 60 and 62°C were used to predict D at 60°C of 15.3 s and 24.3 s when based on survivor CFU in SM 110 and nutrient agar, respectively. The z value was 4.9°C in either medium. D-values for inactivation of S. aureus at 60, 62, 64 and 67°C were used to predict D at 60°C of 41.2 s, 41.0 s and 34.7 s when based on survivor CFU in BP, TSA and TSAS, respectively. The z values were 6.5, 6.5 and 6.4°C, respectively.

A renewed interest in establishment of human milk banks to provide for infants who could possibly benefit from consumption of human milk but cannot be breast-fed exists in the United States (1,13,25). Association of human milk consumption to protection against infection and overall nutritional superiority (14) led to resurgence of interest in human milk banking.

One major concern involved with banking of human milk is the question of whether or not the milk needs to be heat-treated. Bjorcksten et al. (4) suggested that pasteurization of donated breast milk was unnecessary since, in their experience, potential pathogens in the milk normally produced no harmful effects. However, breast milk can contain numerous bacteria which probably originate from the infant's mouth or mother's skin (9,13,24). Bacteria present in breast milk include the potential pathogens Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae (6,9,13,15,24,25). The Committee on Nutrition of the American Academy of Pediatrics (1) advised that extreme caution be used to insure microbiologically safe human milk. They also feel that with the possibilities for bacterial contamination, some form of heat treatment is necessary for banked human milk.

Most previous studies on effects of processing on bacteria in human milk examined long-time heat treatments. Pasteurization at 62.5°C for 30 min eliminates pathogenic bacteria (13). Baum (3) studied thermal destruction of E. coli in human milk exposed to 62.5°C for up to 15 min. However, data on thermal destruction of microorganisms in human milk suitable for use in optimizing pasteurization treatments are not available.

Little information is available on the applicability of high-temperature short-time (HTST) pasteurization procedures to human milk. One reason HTST heat treatments have not been thoroughly investigated as a method of pasteurization for human milk is the lack of a heating apparatus for effective treatment of small volumes of human milk. In fact, Goldblum et al. (10) used a plate heat exchanger designed for use with cow's milk. To achieve sufficient volume to operate the apparatus, pooled human milk was injected into a stream of sterile distilled water. Goldsmith et al. (11) used a HTST heating system designed to operate with as little as 20 ml of human milk. However, both of these studies dealt primarily with the effects of HTST processing on protective factors and micronutrients in human milk. Very little information is available on effects of HTST processing on pathogenic bacteria in human milk.

The present study was designed to assess thermal injury and to determine the rate of thermal destruction of S. aureus in human milk, primarily using HTST pasteurization conditions. These data and similar data for protective factors and micronutrients in human milk (16) will result in selection of HTST treatments that will eliminate pathogens and lower aerobic plate count with minimal damage to nutritional and biological components. Such data are required for development of guidelines for operation of human milk banks.

MATERIALS AND METHODS

Milk samples

Donors were initially contacted 1 d postpartum at two local hospitals, Athens General Hospital and St. Mary's Hospital, Athens, Georgia. Donors were informed of the objectives of...
the study and their role. Those interested in participating were asked to sign a consent form indicating that they understood the project, their role in the project, and that participation was strictly voluntary.

Milk samples were collected by the donors using either manual expression or a breast pump (Happy Family Products, Los Angeles, CA). Donors were provided with sterile polyethylene containers into which milk was collected. Donors were also provided with 70% isopropyl alcohol swabs (Becton-Dickenson, Rutherford, NJ) to wipe the breast and nipple areas before collection of milk samples. Samples were frozen immediately after collection and stored in the frozen state until used in processing studies.

Source of bacteria

*Staphylococcus aureus* 82:1809 was provided by the Centers for Disease Control, Atlanta, Georgia. This organism was used in thermal processing studies where survivor colony forming units (CFU) were enumerated using both a selective medium, *Staphylococcus Medium* 110 (SM110), and a non-selective medium, nutrient agar (NA). It had been isolated from a disease outbreak in a hospital nursery, although the disease outbreak was not necessarily associated with ingestion of contaminated human milk. For thermal injury studies *S. aureus* isolated from fresh, mature (>30 days postpartum) human milk was used. *S. aureus* was isolated and identified using standard procedures (18). Survivor CFUs were enumerated in Baird-Parker medium (BP), Trypticase soy agar (TSA) and Trypticase soy agar with 7.5% salt (TSAS). These media provide for excellent recovery of thermally injured *S. aureus* (2,21).

The same processing procedures were used in all experiments. Composite samples of at least 500 ml of mature human milk containing approximately equal volumes (100-120 ml) of milk from 5 donors were used. Each composite sample was homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at a low speed for 60 s and then divided into five subsamples: one as the unheated control and the remaining four for heat treatments.

Milk samples were inoculated with *S. aureus* at a level of approximately 10^6 CFU/ml of inoculated milk. Each culture was grown in a culture tube containing 10 ml of trypticase soy broth for 14 h in a 30°C water bath. The broth was centrifuged at 3000 × g for 10 min. The supernatant liquid was discarded and the cells were washed twice with 10 ml of 100 mM phosphate buffer, pH 7.2 (18). The washed cell pellet was resuspended in 5 ml of the same phosphate buffer and adjusted to approximately 10^6 CFU/ml with the same buffer by measuring the optical density of the cell suspension using the Hewlett Packard 8451A Diode Array Spectrophotometer (Hewlett Packard, Palo Alto, CA).

A high-temperature short-time (HTST) pasteurizer designed and built specifically for treatment of small quantities of human milk was used to process the samples. The pasteurizer can operate in the range of 30 to 90°C and at flow rates of 5 to 25 ml/min. The heating portion of the apparatus consists of two sections: (a) a heating tube in which milk is rapidly brought from an initial temperature of approximately 10°C to processing temperature and (b) a holding tube in which milk is held at the processing temperature for any desired length of time. Interchangeable holding tubes in a variety of sizes were made available to permit selection of any desired holding time. Holding time is a function of the flow rate through the holding tube which was precisely controlled using a high-pressure diaphragm metering pump (Model 51-1102, American Lewa, Inc., Natick, MA). Processing temperature was accurately controlled by immersing both sections of the heating unit in a Lauda Model C-20B Heating Circulator (Fisher Scientific, Pittsburgh, PA). Residence time in the heating tube ranged from 0.2 s at a flow rate of 25 ml/min to 1.0 s at a flow rate of 5 ml/min. A cooling coil immediately following the holding tube rapidly reduced the temperature to 3-7°C. The jacketed coil is cooled by recirculating cold tap water using a Lauda Model RC-20B Refrigerated Circulator (Fisher Scientific, Pittsburgh, PA).

In some instances, to obtain data at four treatment temperatures, a low-temperature treatment requiring times longer than what is feasible in the continuous system was used. This was accomplished by placing 2-ml portions of milk in preheated test tubes (13 mm × 100 mm) in a constant temperature water bath (Lauda Model C-20B Heating Circulator, Fisher Scientific) and holding for the desired processing time. Treatments used for *S. aureus* included 52°C for 7.5 min and 15 min, respectively. Come-up time was determined by placing a thermocouple in one tube and monitoring temperature until processing temperature was reached. At 52°C come-up time was 2 min. Destruction of bacteria was minimal during the come-up time.

Three trials were completed at each temperature using different composite milk samples. The D-values for *S. aureus* in each medium were determined at four temperatures using four processing times.

Bacteria in each composite milk sample were enumerated before inoculation, after inoculation and after heating. Standard procedures for enumeration of *S. aureus* on SM110, NA, BP, TSA and TSAS were followed (20). Sufficient dilutions were made so that only plates containing between 30 and 300 colony forming units were counted. Duplicate plates were counted in all instances. Plates were incubated at 37°C for 48 h.

Calculation of D and z values

D and z values were calculated by linear regression analysis using the following relationships:

\[
\log \frac{N}{N_0} = -\frac{t}{D} (1)
\]

where \(N_0\) is the bacterial count at time 0 and \(N\) is the bacterial count at time \(t\) and

\[
D = D_o(10)^{(T - T_o)/z} (2)
\]

where \(D_o\) is the reference decimal reduction time at temperature \(T_o\) and \(D\) is the D-value at any temperature \(T\) (22).

Statistical analysis

Each batch of pooled human milk assigned to a given temperature treatment was subdivided into five parts with four parts being randomly assigned to four different processing times and the remaining one part serving as a control. Three replicates were randomly assigned to each temperature treatment.

Statistical analysis of the data required fitting regression equations separately for each temperature treatment since the four levels of processing time were different at various temperatures. Regression estimates of the parameter \(D\) were obtained for each of three replications within each temperature treatment using the general linear model procedure (GLM) described by Ray (17). Parameter estimates of \(z\) were determined using the same regression procedures and using all three estimates of \(D\) within each temperature treatment. Paired comparisons were completed using procedures from Snedecor and Cochran, 1980 (19).
RESULTS AND DISCUSSION

D-values at various temperatures for *S. aureus* 82:1809 in human milk plated on SM110 and NA after heating are shown in Table 1. Predicted values of D at 52, 58, 60 and 62°C were 679.2, 39.6, 15.3 and 5.9 s, based on CFUs on SM110. When based on CFUs on NA, D values were 1059.7 s at 52°C, 62.5 s at 58°C, 24.3 s at 60°C and 9.5 s at 62°C. The z value for *S. aureus* was 4.9°C with both types of media (Table 1).

D-values for *S. aureus* based on CFUs on NA tended to be higher than those calculated from CFUs on SM110. This suggests that sublethal injury to *S. aureus* occurs. To further assess the degree of thermal injury to *S. aureus*, pre-heated (72°C, 33 s) human milk samples were inoculated with *S. aureus* and pasteurized at 64°C for 33 s at all temperatures. Paired comparisons showed that pre-heated milk may affect the accuracy of D-values based on CFUs on selective media. In addition, active cellular material present in human milk may be damaged by a pre-heat treatment. Thus, while thermal injury may influence accuracy of D values based on CFU on selective media SM110 data, destruction of protective factors in pre-heated milk may affect the accuracy of D-values based on CFU on NA. These observations suggest that data from a more selective and diagnostic medium which also allows excellent recovery of injured cells were needed to accurately assess D- and z-values for *S. aureus* in raw human milk. For this purpose, BP, TSA, and TSAS were chosen as the recovery media in assessing the thermal injury of *S. aureus*. The isolate of *S. aureus* native to human milk was used for the following study instead of *S. aureus* 82:1809.

Table 3 shows the predicted D-values at various temperatures for *S. aureus* in human milk on BP, TSA and TSAS. The BP medium showed higher recovery of heat-processed *S. aureus* at all temperatures. Paired comparisons showed that BP was significantly different (P<0.01) from more selective and diagnostic media which also allows excellent recovery of injured cells were needed to accurately assess D- and z-values for *S. aureus* in raw human milk. For this purpose, BP, TSA, and TSAS were chosen as the recovery media in assessing the thermal injury of *S. aureus*. The isolate of *S. aureus* native to human milk was used for the following study instead of *S. aureus* 82:1809.

### Table 1. D-values at various temperatures and z values for *Staphylococcus aureus* in human milk on *Staphylococcus Medium 110* (SM110) and nutrient agar (NA).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Temperature (°C)</th>
<th>D-value (s)</th>
<th>z-value (°C)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM110</td>
<td>52</td>
<td>679.2</td>
<td>4.9</td>
<td>0.9948</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>39.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>15.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>5.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>52</td>
<td>1059.7</td>
<td>4.9</td>
<td>0.966</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>62.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>24.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>9.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Predicted value based on linear regression analysis.

### Table 2. Comparison on *Staphylococcus Medium 110* and Nutrient Agar for *Staphylococcus aureus* in human milk.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SM110 (CFU/ml)</th>
<th>NA (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-heated milk</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Inoculated pre-heated milk</td>
<td>1.3 \times 10^6</td>
<td>1.4 \times 10^6</td>
</tr>
<tr>
<td>64°C, 33 s</td>
<td>0</td>
<td>1.1 \times 10^3</td>
</tr>
<tr>
<td>58°C, 80 s</td>
<td>4.1 \times 10^4</td>
<td>3.2 \times 10^4</td>
</tr>
</tbody>
</table>

* *S. aureus* 82:1809

* Bacterial counts are means from 3 composite milk samples.

### Table 3. D values at various temperatures and z values for *Staphylococcus aureus* in human milk on *Baird-Parker Medium* (BP), *Trypticase soy agar* (TSA) and *Trypticase soy agar with 7.5% NaCl* (TSAS).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Temperature (°C)</th>
<th>D-value (s)</th>
<th>z-value (°C)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>60</td>
<td>41.2</td>
<td>6.5</td>
<td>0.9954</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>20.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>10.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSA</td>
<td>60</td>
<td>41.0</td>
<td>6.5</td>
<td>0.9952</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>20.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>9.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSAS</td>
<td>60</td>
<td>34.7</td>
<td>6.4</td>
<td>0.9999</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>16.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>8.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* An isolate from human milk.

* Predicted value based on linear regression analysis.
from either TSA or TSAS in its ability to recover thermally injured \textit{S. aureus} from human milk (12). For TSA, D-values were somewhat lower but very close to those of BP. The D-values of \textit{S. aureus} based on CFU on TSAS were much lower than D-values on either BP or TSA at all temperatures. The predicted D-values based on CFU on TSAS at 60, 62, 64, and 67°C were 34.7 s, 16.8 s, 8.2 s, and 2.8 s, respectively.

Baird-Parker medium is a highly specific diagnostic and selective medium for isolation and enumeration of coagulase-positive staphylococci. There are two constituents in BP medium which help in recovering heat-shocked \textit{S. aureus}; egg yolk tellurite acts as a protective agent (21) and sodium pyruvate destroys peroxides which are harmful to the sublethally injured \textit{S. aureus} (2). TSA is a general purpose medium for cultivation of fastidious microorganisms (7) which may provide some protection for the recovery of the injured \textit{S. aureus} although not as much as BP. The high concentration of sodium chloride in TSAS however resulted in suppression of heat-shocked \textit{S. aureus} (5). Comparison of D-values of five media, SM110, NA, BP, TSA and TSAS, showed that the recovery of thermal injured \textit{S. aureus} was best on BP followed by TSA, TSAS, NA, and SM110.

The thermal destruction curve used to determine the \(z\)-value for \textit{S. aureus} in human milk using BP as the recovery medium is shown in Fig. 1.

Very few data are available on thermal destruction of \textit{S. aureus} in human milk. Eyres et al. (8) reported that all \textit{S. aureus} in human milk were destroyed after pasteurization of 62.5°C for 30 min with an initial level of \(2.8 \times 10^7\) CFU/ml. Based on the regression equation in Fig. 1, the D-value for \textit{S. aureus} at 62.5°C would be 17.04 s; therefore, a 30-min treatment would be expected to be sufficient to eliminate \(2.8 \times 10^7\) CFU/ml. Lucas and Roberts (13) showed that pasteurization of human milk at 62.5°C for 30 min eliminated \textit{S. aureus}. Although the initial load was not specified, this treatment would be expected to be sufficient based on the predicted D-value of 20.4 s at 62°C in Table 3.

Reported D- and \(z\)-values for \textit{Staphylococcus} spp. in acid and pasteurized foods at 82.2°C are 0.4 s and 7°C, respectively (23). The predicted D-values at 82.2°C in this study were 0.017 s, 0.015 s, 0.011 s and \(z\)-values were 6.5, 6.5 and 6.4°C on BP, TSA and TSAS, respectively. The \(z\)-values in these two studies are very close. The differences in D-value are probably due to strain variations or compositional differences of the foods in which D values were determined.

The kinetic parameters established in this study, when coupled with similar data for other pathogens likely to occur in human milk and for protective factors and micronutrients in human milk, can be used to calculate an optimum process for pasteurization of human milk. Such a process would insure destruction of pathogens, yet maximize retention of protective factors and micronutrients. This information is needed for establishment of guidelines for operation of human milk banks.


Lin, et al., con't. from p. 672


