A Research Note

Immunological Evaluation of Pasteurized, Deaerated Human Milk

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ABSTRACT

Retention of immunoglobulin A (IgA) and IgA specific for enteropathogenic Escherichia coli (EPEC) was determined in human milk that was processed and stored under refrigeration or frozen conditions. Human milk was subjected to three different processing conditions: (a) deaerated, vacuum packaged in metalized polyester bags and pasteurized (64°C for 6 min, 10 s); (b) vacuum packaged and pasteurized; (c) vacuum packaged. Samples were stored at 4°C for 7 d and at -20°C for 28 d. An enzyme-linked immunosorbent assay (ELISA) to detect antibodies to EPEC was developed and used to monitor these antibody levels, while radial immunodiffusion (RID) plates were used to provide information on total IgA of the milk samples. In addition, the dissolved oxygen content of the samples was monitored. It was found that pasteurization and deaeration did not adversely affect the IgA levels, and levels of total IgA and IgA specific for EPEC remained stable or increased slightly during storage in most samples regardless of the storage temperatures.

Human milk is ideal for the nutritional and immunological needs of infants. Transmission of passive immunity is largely prenatal in primates, but additional protective factors are present in human milk which protect infants from infection (2). Of these, immunoglobulin A (IgA) is found in greatest concentration (14). Conditions may exist, however, which prevent a mother from providing milk for her own infant. Human milk banks can provide adequate and reliable supplies of human milk (8,10,21).

Collected breast milk is often contaminated with bacteria (13). The microbiological safety of banked human milk is enhanced by proper collection and processing techniques, including pasteurization (1). Unfortunately, heat treatments severe enough to eliminate or significantly reduce microbial hazards may also destroy some of the protective factors (5,6,11).

Deaeration is known to increase stability of oxygen labile nutrients (12). Removal of oxygen may be performed without causing a shift in the population of surviving microorganisms toward pathogenic varieties which are microaerophilic or anaerobic (16). The effect dissolved oxygen has on protective factors such as IgA during processing and storage of human milk has not been determined.

Standards have not been established for processing and storing banked human milk. This study was designed to determine the effect deaeration, pasteurization, and storage have on protective factors such as IgA. Processing conditions used were based upon previous work using the same water bath temperature and packaging material which resulted in 8 D and 10 D reductions in Staphylococcus aureus and Escherichia coli, respectively (18). Retention of total IgA was determined by radial immunodiffusion (RID), while IgA specific for enteropathogenic E. coli (EPEC) was evaluated by means of an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Collection, handling, and processing of human milk samples

Mature (> 1 mo, < 8 mo postpartum) human milk samples were collected from donors living in or around Athens, GA. Milk was manually expressed into sterile high density polyethylene bottles in the donors’ homes, frozen, collected by laboratory personnel, and maintained at -20°C until processed. Prior to processing, milk samples were thawed separately at 4°C and homogenized using a Polytron Homogenizer (Brinkman Instruments, Westbury, NY) at a low speed for one minute.

The effectiveness of the ELISA that was developed in measuring IgA specific for EPEC was determined by analyzing milk from individual donors for the immunoglobulin immediately after processing. The ELISA was also used to measure the retention of EPEC specific IgA in milk composites that were processed and then stored. The composites were composed of milk from five donors.

Samples were either pasteurized and deaerated (PD), pasteurized and not deaerated (PNOD), or neither pasteurized nor deaerated (NPNOD) as described by Musgrove et al. (16) except that pasteurization time and temperature were modified to 64°C and 6 min, 10 sec. Processing conditions included allotments for come-up and come-down time as established by Sherman (18).

Samples that were stored at 4°C were analyzed over 7 d while those stored at -20°C were analyzed over 28 d.
Dissolved oxygen content

Dissolved oxygen content was determined as described by Musgrove et al. (16).

Radial immunodiffusion assay (RID)

Total IgA was quantified by the RID techniques described by Morgan et al. (15) using prepared RID plates and standards (LC-Partigen IgA Kit; Behring Diagnostics Inc., Somerville, NJ). Samples were diluted 1:5 and assayed in triplicate.

Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA technique based on the procedure of Engvall and Perlmann (4) was used to determine the presence of IgA specific for various strains of enteropathogenic E. coli (EPEC) in raw and processed human milk samples. Six strains of EPEC (055:H7, 086:Nm, 0111ab:H21, 0119:H6, 0125ac:Nm, 0128ab:H2) implicated as causative agents of infant diarrhea were obtained from the Centers for Disease Control in Atlanta, GA. Each strain was cultured separately in trypticase soy broth (Difco, Detroit, MI) at 37°C for 48 h. Cells were harvested by centrifugation at 15,000 RPM for 10 min at 4°C and washed with buffered phosphate diluent (BPD; 0.2 M, pH 7.2). The antigen preparation was obtained by boiling 1 g of cells in 99 ml of BPD for 15 min. After cooling to room temperature, the suspension was diluted 1:10 in a buffer composed of equal portions of 0.01M di-sodium phosphate and 0.15M sodium chloride. All other reagents for the ELISA were obtained as an ELISA kit (KPL, Inc., Gaithersburg, MD).

Flat-bottomed 96-well polystyrene microtitration plates (Linbro/Titertek, Flow Labs, McLean, VA) were used as the solid-phase carrier. Wells were coated with 100 µl of the antigen preparation of each strain or a mixture of the six and incubated overnight at ambient temperature. Plates were used immediately or stored at 4°C. The antigen preparation was removed, and the wells coated with 100 µl BSA diluent/blocking solution (incubated at room temperature for 5 min, then emptied) prior to addition of 100 µl portions of processed milk (PD, PNOD, NOPNOD). Plates were incubated for 30 min at room temperature. Negative control wells were coated with antigen preparation and received no milk samples, or wells were not coated with antigen preparation but received milk samples. Triplicate wells were prepared for each sample. Wells were washed four times with a solution of buffered saline and Tween 20 after milk samples were removed. Each well received 100 µl of goat antihuman IgA(a) conjugated to peroxidase and was incubated 30 min at room temperature. Plates were emptied of antibody solution, soaked for 5 min in the wash solution, and washed as described previously. Wells then received 100 µl of substrate solution composed of equal portions of hydrogen peroxide and 2-2 azino-di-3-ethyl benzothiazolin sulfone-6 (ABTS). A peroxidase stop solution was added, and the color intensity was quantified by spectrophotometric measurement (405 nm) using an automatic plate reader (Titertek Uniskan, Helsinki, Finland).

RESULTS

The ELISA detected IgA specific for EPEC consistently for each strain of E. coli tested (Fig. 1), and there was no significant difference (p<0.05) between treatments for each strain. Human milk IgA specific for EPEC was also determined to be stable during storage after processing (Table 1). At 4°C storage, slight increases in concentration were observed up to day 7, while during -20°C storage, increases were observed up to day 21.

Immunoglobulin A levels measured by RID in treatments PNOD and NOPNOD stored at 4°C increased slightly throughout storage, while the levels in PD treated samples decreased after 4 d (Table 2). In samples stored at -20°C, the amount of total IgA stayed approximately the same during storage. Other than the differences noted in samples stored at 4°C on day 7, there were no noticeable differences observed due to processing.

The lowest concentration of dissolved oxygen was in samples which were deaerated (Table 3). Samples which were deaerated had slightly greater levels of specific IgA to EPEC as determined by ELISA (Table 1).

DISCUSSION

In this study, IgA specific for EPEC was found to be stable to the processing and storage treatments. The increase in EPEC specific IgA contents detected by ELISA is similar to observations made concerning total IgA (9,17,20). The increase in IgA detected during storage of breast milk samples may be due to the release of leukocytes into the sample from the pouch surfaces and the continued release of immunoglobulin from the leukocytes over time. Freezing may cause cells to be released from the container walls, while the mild heat treatment used.
TABLE 3. Dissolved oxygen content of human milk stored in flexible, metalized polyester pouches. Milk was either pasteurized (64°C for 6 min, 10 sec) and deaerated (PD), pasteurized but not deaerated (PNOD), or neither pasteurized nor deaerated (NOPNOD). Values are the means of three measurements and are expressed as mg IgA/dl.

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<th>Treatment</th>
<th>Days at 4°C</th>
<th>Days at -20°C</th>
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<tr>
<td></td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>PD</td>
<td>26.0</td>
<td>36.2</td>
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<tr>
<td>PNOD</td>
<td>26.0</td>
<td>31.2</td>
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<td>NOPNOD</td>
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may be sufficient to free compartmentalized immunoglobulin yet mild enough so that its denaturation did not occur. Gaffin et al. (7) also observed an increase in the quantity of IgA detected following processing.

The milder pasteurization temperature used in this study may have been harsh enough to break apart the secretory IgA dimer without destroying the actual IgA molecule. Secretory IgA differs from IgA in that it exists as a dimer, two IgA molecules joined together by a secretory piece (19). This breakage could enable the immunoglobulin to bind with less steric hindrance in the assay and thereby increase its detection.

Radial immunodiffusion results for total IgA in stored human milk samples did not follow the same pattern of detection as the indirect ELISA for EPEC specific IgA. Goldblum et al. (8) made a similar observation, indicating that specific IgA was affected without detecting a difference in total or secretory IgA. Cruz and Arevalo (3) found that levels of specific antibody fluctuated and were detected independently of milk secretory IgA.

In summary, the combination of pasteurization parameters, inherent protective components such as fat, preservation of the fat emulsion through homogenization, use of metalized polyester containers, and freezing of samples before and after processing conspires to release compartmentalized IgA and minimize the destruction of soluble IgA. This resulted in increased detection of this immunoglobulin in human milk samples. Pasteurization conditions severe enough to reduce the numbers of potentially pathogenic microorganisms followed by storage do not adversely affect levels of IgA specific to EPEC in human milk.

TABLE 2. Radial immunodiffusion plate results for IgA in human milk that had been pasteurized (64°C; 6 min, 10 sec) and deaerated (PD), pasteurized but not deaerated (PNOD), or neither pasteurized nor deaerated (NOPNOD). Values are the means of three measurements and are expressed as mg IgA/dl.

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ACKNOWLEDGMENTS

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REFERENCES