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Quantification of Bovine IgG in Milk Using Enzyme-linked Immunosorbent Assay

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A double sandwich enzyme-linked immunosorbent assay (ELISA) procedure for the quantification of IgG in bovine milk was developed for detecting the concentration of IgG in various homogenized HTST, UHT, evaporated and raw milk samples as well as skim milk powder. ELISA results were consistently lower than radial immunodiffusion (RID) values for the same sample, suggesting interference by an unknown constituent in the milk matrix. This difficulty was overcome by using a reference milk, quantified previously for IgG by RID. A significant linear relationship ($y = 2.03x - 0.034$, $r = 0.891$, $n = 150$, $p < 0.001$) was obtained for IgG content in unknown raw milk samples determined by ELISA using serial dilutions of the reference milk (y) or commercial IgG (x) to construct standard curves. The slope of the regression line could be used as a correction factor. Alternatively, IgG in unknown samples could be directly quantified from absorbance values of reference milk serial dilutions assayed on the same ELISA plate. Using this procedure, homogenized, HTST pasteurized milk contained from 65 to 79% of the IgG found in raw milk. Skim milk powder also retained a major portion of IgG, while evaporated and UHT pasteurized milk were virtually devoid of IgG.

Keywords: bovine milk, ELISA, IgG.

INTRODUCTION

IgG is a major immunoglobulin in bovine milk and is involved in secondary responses to previously encountered antigens. The immunological activity of bovine immunoglobulin in colostrum from hyper-immunized cows is similar to human milk IgG and has been recommended for inclusion in infant formula (Hilpert *et al.*, 1977). Clinical studies have demonstrated the important potential role of using milk IgG in preventing mortality of infants exposed to infectious diseases (Narayanan *et al.*, 1983). Oral administration of 'immune' milk derived from cows immunized with non-viable pathogens has recently been shown to protect against intestinal bacteria infections

(Hilpert *et al.*, 1977; Kobayashi *et al.*, 1991). Other studies have shown a significant efficacy of bovine milk IgG from non-immunized cows in preventing gastro-intestinal diseases in infants (Ballabriga, 1982). In addition to colostrum, sweet whey has been identified as an important potential source of immunoglobulin and other anti-microbial agents such as lactoferrin and lysozyme (Kaneko *et al.*, 1985; Al-Mashikhi & Nakai, 1987).

The use of milk from either immunized or non-immunized cows as a source of IgG requires a sensitive method for routine screening of milk IgG that is relatively simple, time efficient, and relatively inexpensive to perform. Non-immunological methods used for IgG identification include analytical ultracentrifugation (Normansell & Stanworth, 1966), electrophoresis (Larson, 1957; Kaneko *et al.*, 1985) and turbidity measurements using a colostrometer (Fleenor & Strott, 1980). Immunological methods developed for Ig detection in milk include double radial (Guidry *et al.*, 1980; Caffin *et al.*, 1983) and single (Penhole & Christie, 1969; Newstead, 1976; Al-Mashikhi & Nakai, 1987) immunodiffusion, immuno-electrophoresis (Watson *et al.*, 1972; Al-Mashikhi & Nakai, 1987), immuno-nephelometry (Montagne *et al.*, 1991) and indirect or sandwich enzyme-linked immunosorbent assay (Akerlund *et al.*, 1977; Loeffler & Norcross, 1985; Smith *et al.*, 1989).

The enzyme-linked immunosorbent assay (ELISA) procedure is a sensitive method for measuring antibodies to a wide range of antigens and involves the use of stable and relatively inexpensive reagents. The relative simplicity and ability to detect antibodies of specific immunoglobulin class makes ELISA a potentially useful screening method for detecting IgG from non-immunized milk sources. The purpose of the present study was to develop and standardize an ELISA test for the quantitative detection of IgG in milk.

MATERIALS AND METHODS

Reagents

Chicken egg ovalbumin (Grade V), rabbit anti-bovine IgG (whole molecule) alkaline phosphatase conjugate, alkaline *p*-nitrophenyl phosphate substrate (*p*-NPP), agarose (type IV), bovine IgG and Tween-20 were purchased from Sigma (St Louis, MO, USA). Rabbit anti-bovine IgG (H+L) antiserum was obtained from Miles Scientific (Naperville, IL, USA). ELISA microtitre plates were purchased from Fisher Scientific (Ottawa, ON, Canada).

Buffers

Buffers included the carbonate coating buffer at pH 9.6 (1.59 g Na₂CO₃; 2.93 g NaHCO₃; 0.2 g NaN₃; 1 l H₂O), phosphate-buffered saline-Tween (PBS-Tween) at pH 7.4 (8.0 g NaCl; 0.2 g KH₂PO₄; 1.15 g Na₂HPO₄; 0.2 g KCl; 0.2 g NaN₃; 0.5 ml Tween 20; 1 l H₂O), blocking buffer (0.25% ovalbumin in PBS-Tween) and a 10% diethanolamine (DEA) buffer (97.0 ml diethanolamine; 0.1 g MgCl₂·6H₂O; 0.2 g NaN₃; concentrated HCl to pH 9.8; H₂O to 1 l).

Milk Samples

Raw milk was collected immediately after milking from the University of British Columbia dairy farm. Raw milk samples were also obtained from regional dairies through the provincial milk testing laboratory (J. R. Laboratories, Burnaby, BC, Canada). Commercial HTST pasteurized and UHT sterilized fluid milk, canned evaporated milk and skim milk powders were purchased from local supermarkets.

100 μl (1 $\mu\text{g ml}^{-1}$) anti-bovine IgG antiserum in carbonate coating buffer and incubated for 60 min at 37°C. After washing the wells twice with PBS-Tween, 250 μl of blocking solution was applied to wells for 30 min at 37°C. The blocking solution was removed and 100 μl sample or IgG standard was added to the wells. Bovine IgG standards and milk samples spiked with IgG were diluted in PBS-Tween to contain IgG between 1 and 100 ng ml^{-1} and milk was diluted between 1000 to 30 000-fold. ELISA plates were incubated again for 60 min at 37°C. After washing three times with PBS-Tween, 100 μl conjugate (anti-bovine IgG alkaline phosphatase conjugate diluted to 0.43 $\mu\text{g ml}^{-1}$ in PBS-Tween) was added to each well and microtitre plates were incubated for 60 min at 37°C. Wells were washed three more times with PBS-Tween and given a final rinse with distilled water before addition of 100 μl substrate (0.5 mg *p*-NPP ml^{-1} DEA buffer). After a 60 min incubation at 37°C the yellow colour developed by conversion of the substrate was measured at 405 nm, using a reference filter at 655 nm with a Bio-Rad ELISA plate reader (model 450, Bio-Rad, Richmond, CA, USA). Standards and samples were assayed at least in triplicate. Absorbance values from ELISA plates were transformed, using the equation $y=(ax/b+x)+c$ where y =absorbance and x =concentration, to IgG concentrations using the computer program ELISA (ELISA release 2.79; Leading Edge Research, Vancouver, BC, Canada).

Radial Immunodiffusion (RID)

Agarose (2% (w/v) in 0.01 M-PBS, pH 7.4) was brought to boiling then cooled to 52°C before adding to the antisera. A 5 ml aliquot of the agarose solution held in a 47°C water bath was then added to 2 ml of antiserum to give a final concentration for rabbit anti-bovine IgG in PBS of 71.4 $\mu\text{g ml}^{-1}$. Antisera and agarose were gently mixed by swirling in a warm (47°C) water bath. All bubbles were removed before pouring the mixture into level polystyrene RID plates (ICN Biochemicals, St Laurent, Que, Canada). Sixteen 3 mm holes were punched into the gel where 3 μl each of standard and sample were added. Ring size was measured after 18 and 24 h incubation at ambient temperature, using a Mauser micrometer (Scherr-Tumico, Los Angeles, USA).

Milk and Standard Preparations

Concentration of a stock solution of commercial IgG diluted to approximately 1 mg ml^{-1} in PBS buffer was determined by spectrophotometry. An extinction coefficient ($E_{1\%}^{1\text{cm}}$) of 13.7 at a wavelength of 280 nm was used. IgG concentration in a fresh raw milk sample was assayed by RID, using the spectrophotometrically measured IgG as the standard. This milk sample was used as a reference milk for subsequent ELISA analysis of other milk samples.

For determining IgG recovery in milk, commercial bovine IgG was added to pasteurized homogenized milk and a standard IgG curve with values ranging from 1.0 to 20.0 ng ml^{-1} was prepared.

Skim milk was prepared from raw whole milk by removing the cream layer obtained after centrifugation at ambient temperature. Whey fractions were prepared from the skim milk by acidification to pH 4.6 with 1 M-HCl and incubated at 37°C for 15–30 min. The acid-precipitated casein was removed by centrifugation at ambient temperature. Representative aliquots of raw whole milk, skim milk and whey were further dialysed for 24 h at 4°C using dialysis tubing having a molecular weight cut-off of 6000–8000. The dialysed and non-dialysed milk and whey samples were analysed for total IgG content using both ELISA and RID.

RESULTS

The detection of IgG in milk by ELISA was investigated at four concentrations of

antisera ranging from $0.1 \mu\text{g ml}^{-1}$ to $10 \mu\text{g ml}^{-1}$, as shown in Figure 1. The minimal concentration of antiserum required for the greatest sensitivity of IgG detection in milk was $1 \mu\text{g ml}^{-1}$. This optimal amount of coating antibody is far less than that required for RID analysis.

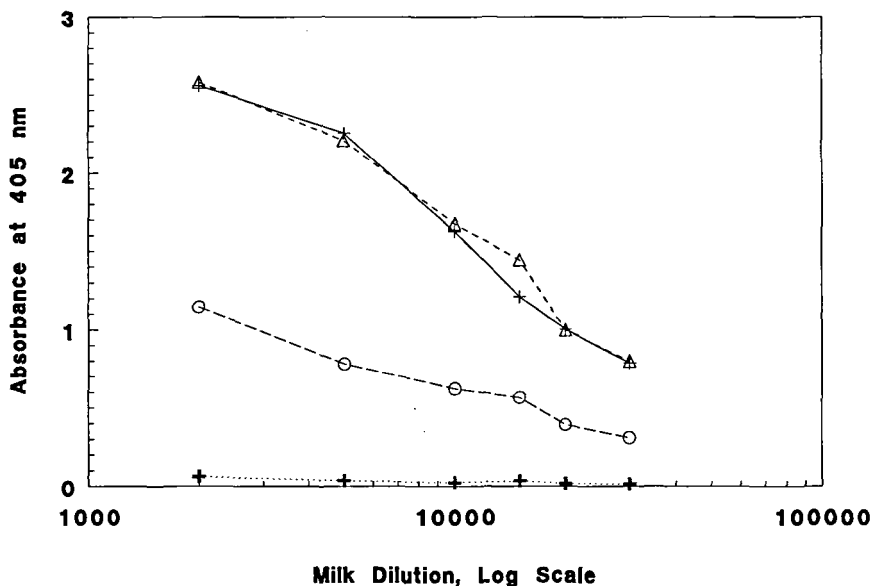


FIG. 1. Optimal antibody coating concentrations in sandwich ELISA IgG test. Coating antibody concentration: $10 \mu\text{g ml}^{-1}$ ($\Delta\Delta\Delta$); $1 \mu\text{g ml}^{-1}$ ($\Delta\Delta\Delta$); $0.1 \mu\text{g ml}^{-1}$ ($\circ\circ\circ$); $0.01 \mu\text{g ml}^{-1}$ ($+++$).

ELISA standard curves obtained for IgG standard in buffer and intrinsic IgG in diluted milk are shown in Figure 2. The two standard curves were not comparable in terms of absorbance values at similar IgG concentrations. The absorbances measured for IgG standards were higher than those measured in milk dilutions, reflecting the presence of an unknown suppressing factor associated with the milk matrix.

The IgG content in various milk fractions analysed by both ELISA and RID is given in Table 1. The distribution of IgG following removal of fat and casein is evident in Table 1. Essentially, all of the IgG remained in the skim milk portion and specifically in the whey portion of milk. Self-creaming, centrifugation and dialysing milk did not improve IgG quantification by ELISA. The interference was common to all milk fractions analysed. It is apparent therefore that extracting milk samples to remove fat and casein prior to performing the ELISA was not sufficient to remove the factor in milk that suppressed IgG detection by ELISA.

The effects of the non-ionic detergent Tween or the ionic strength of the buffer used for sample dilution on ELISA assay values of IgG in milk were investigated. When Tween was excluded, ELISA values were markedly reduced, the absorbance values being only 50–85% of those assayed in the presence of Tween. However, no differences were observed between 0.05, 0.1 and 0.2% levels of Tween. Improvement in the accuracy of an ELISA procedure for analysis of lactoferrin by inclusion of 4 M-NaCl was reported by Meisel (1990). However in the present study, no difference was

TABLE 1. Measurement of IgG in milk fractions by ELISA and RID

Raw milk fraction	IgG concentration (mg ml ⁻¹)		
	RID ^a	ELISA ^b	RID/ELISA
1. Whole milk			
Dialysed	0.302 ± 0.027	0.145 ± 0.004	2.08
Non-dialysed	0.325 ± 0.019	0.147 ± 0.019	2.21
2. Skim milk			
Dialysed	0.313 ± 0.038	0.142 ± 0.002	2.20
Non-dialysed	0.306 ± 0.038	0.156 ± 0.021	1.96
3. Whey			
Dialysed	0.295 ± 0.023	0.123 ± 0.021	2.39
Non-dialysed	0.294 ± 0.026	0.161 ± 0.015	1.83

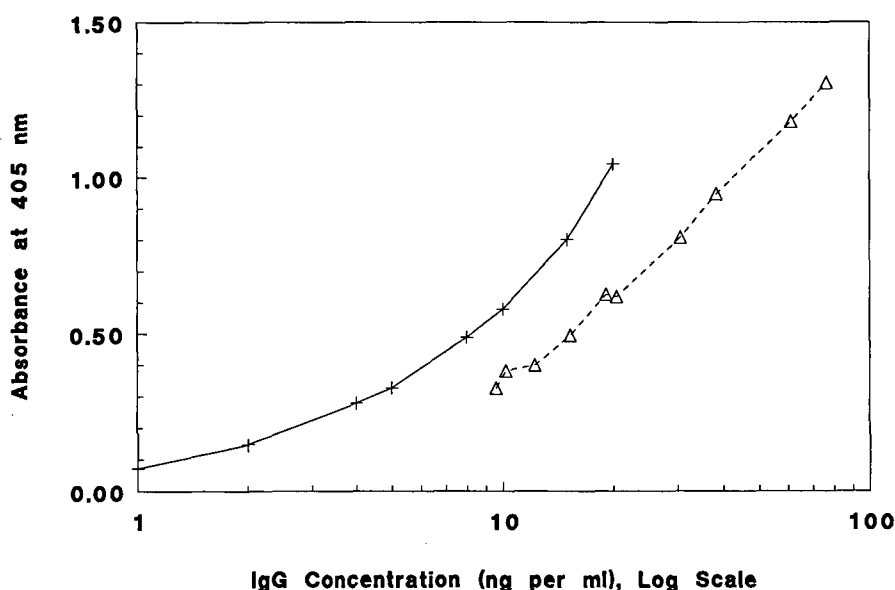
^aValues represent mean from three dilutions.^bValues represent mean from four dilutions in duplicate or triplicate.

FIG. 2. Sandwich ELISA IgG standard curves in milk (ΔΔΔ) and buffer (+ + +) matrices.

observed between values for IgG in milk assayed using PBS-Tween buffer containing 0.14 M- and 4 M-NaCl.

The interference attributed to the milk matrix in quantifying IgG was further studied by comparing IgG standard calibration curves in buffer and in two different dilutions of milk samples (Figure 3). All three calibration curves were parallel (slope=0.025) and showed linearity ($r \geq 0.990$) over a wide range of IgG concentrations. The interference of unknown factors in milk restricting accurate measurement of IgG by ELISA is further shown in Figure 4. Milk spiked with varying amounts of IgG gave consistently lower IgG estimates than predicted, calculated IgG values.

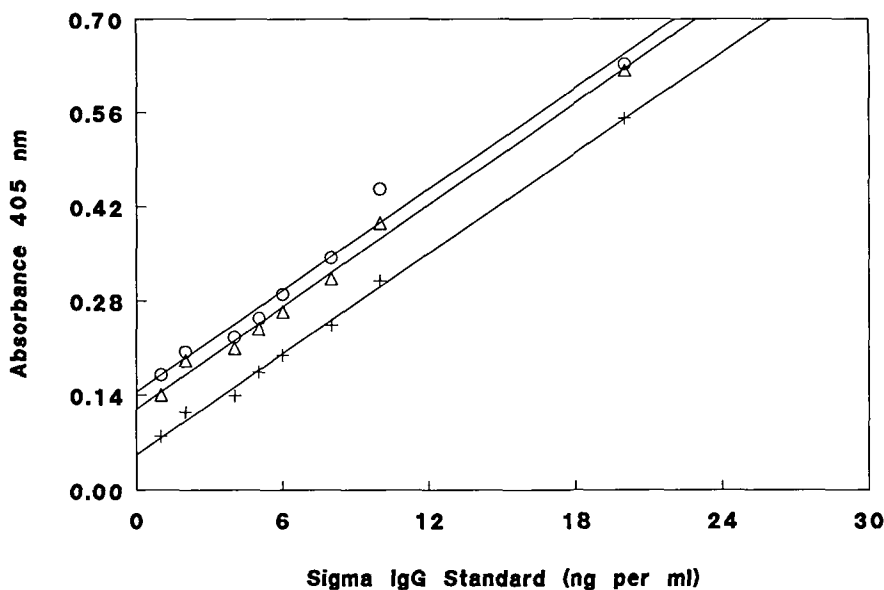


FIG. 3. Sandwich ELISA response for bovine IgG standard in buffer (++++), in milk diluted 1:10000 (O O O) and in milk diluted 1:16000 (Δ Δ Δ).

A method to overcome the suppressing effect of milk constituents in underestimating IgG concentrations by ELISA was developed by first determining the IgG content in a reference milk by RID and in a bovine IgG stock solution by absorbance at 280 nm. The ELISA absorbance values of dilutions of this reference milk and of the stock IgG solution were then measured to construct standard curves for the quantification of IgG in unknown milk samples. A plot of the IgG content calculated from reference milk standard curve versus a pure IgG standard curve is presented in Figure 5. Content of IgG in milk samples determined using reference milk was related to content determined using standard IgG in buffer by the equation $y = 2.03x - 0.034$, with a correlation coefficient of $r = 0.891$ ($n = 150$).

This relationship could be used to calculate corrected values of IgG concentration in unknown milk samples when assayed by ELISA using commercial or pure IgG standards. Alternatively, IgG concentration of unknown milk samples can be directly obtained by including serial dilutions of the reference milk on each ELISA assay plate. The latter procedure would be more accurate due to the day-to-day and plate-to-plate variations which are common to ELISA methods.

The corrected, IgG content in various processed milks measured by the ELISA procedure is presented in Table 2. HTST-pasteurized carton and bottled milks (samples 1, 3, 4 and 5) contained IgG in a range 0.14–0.24 mg ml⁻¹. Pasteurized milk collected from extensive HTST treatment (sample 2) contained lower IgG (0.07 and 0.04 mg ml⁻¹), while only trace amounts of IgG were found in UHT processed milk (sample #9). Moreover, samples of canned evaporated milk (samples 6–8) contained no IgG as compared to the higher IgG (0.166 mg ml⁻¹) in the skim milk powder sample (#10).

TABLE 2. IgG content of some commercial milk samples measured by ELISA^a

Milk processor ^b	Treatment	IgG concentration, mg ml ⁻¹ (mean $\pm$ SD)	Relative % ^c
A1	Homogenized, HTST pasteurized	0.200 $\pm$ 0.009	64.9
A2	Homogenized, HTST pasteurized	0.184 $\pm$ 0.006	59.7
B1	Homogenized, HTST pasteurized	0.071 $\pm$ 0.007	23.1
B2	Homogenized, HTST pasteurized	0.040 $\pm$ 0.009	12.9
C	Homogenized, HTST pasteurized	0.144 $\pm$ 0.011	46.8
D	Homogenized, HTST pasteurized	0.243 $\pm$ 0.017	78.9
E	Homogenized, HTST pasteurized	0.185 $\pm$ 0.007	60.5
F	Evaporated	0.000 $\pm$ 0.000	0.00
G	Evaporated, 2%	0.000 $\pm$ 0.000	0.00
H	Evaporated, 2%	0.000 $\pm$ 0.000	0.00
I	UHT sterilized, 2%	0.003 $\pm$ 0.004	0.65
J	Skim milk powder	0.166 $\pm$ 0.020	53.9

^aValues are corrected IgG, measured from three dilutions in triplicate.

^bEach letter represents a different milk processor. Two different lots were analysed from processors A (A1 and A2) and B (B1 and B2).

^cRelative to our non-homogenized reference raw milk sample with a mean IgG concentration of 0.308 mg ml⁻¹.

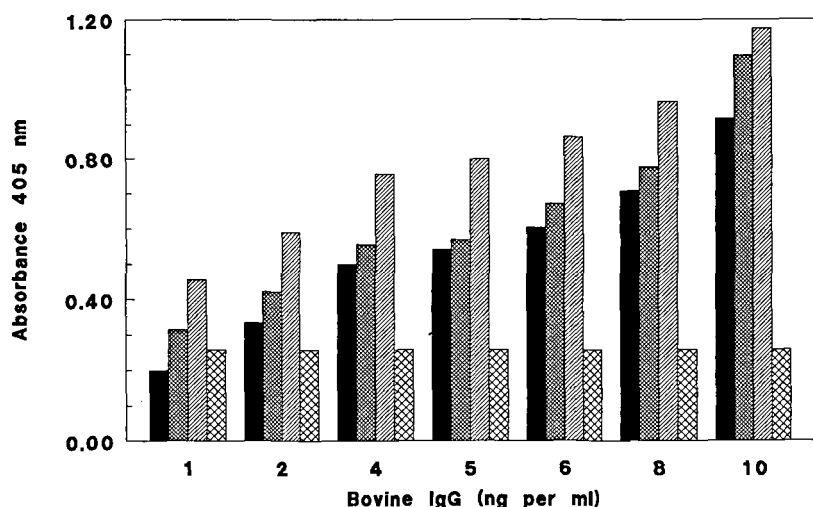


FIG. 4. Sandwich ELISA response for IgG in buffer, milk and spiked milk samples. The x-axis represents the concentration of IgG added to buffer or spiked milk. Samples: IgG in buffer (■); measured milk + IgG (▨); predicted milk + IgG (▧); and milk alone (1:10000) (▩).

DISCUSSION

Several difficulties were encountered that influenced the accurate measurement of bovine milk IgG by ELISA. There is considerable potential for using ELISA for routine testing of IgG content in milk, since daily sample volume turnover is much greater and the amount of antiserum required is far less than standard RID methods. However, the immunodiffusion assay gave constantly higher values of IgG than ELISA and agreed with suggested literature values. We report that an unknown factor present in milk suppresses total IgG detection in ELISA compared to RID analysis. The

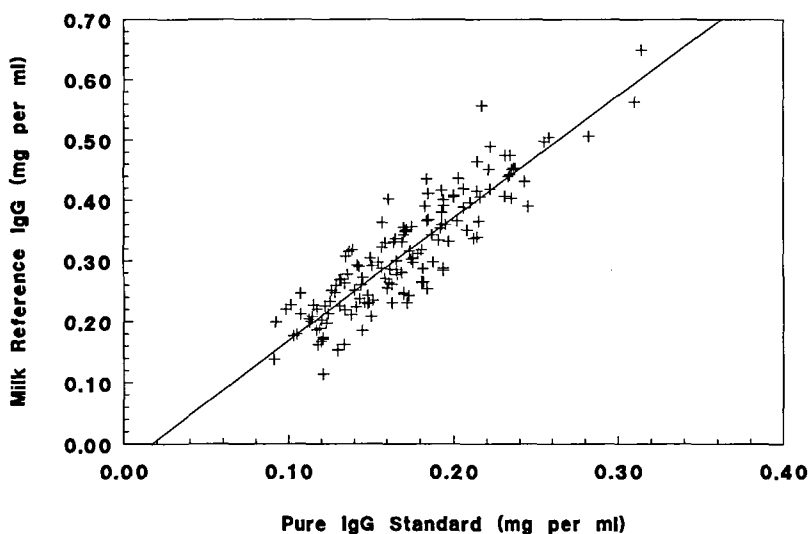


FIG. 5. Relationship between IgG content in raw milk samples determined by sandwich ELISA using either commercial bovine IgG (x-axis) or a reference milk (y-axis) as standard curves. IgG contents of commercial IgG and the reference milk used to construct standard curves were independently assayed using absorbance at 280 nm and RID, respectively.

reason for the quantitative discrepancy between ELISA and RID remains unknown, however a number of suggestions are given and a final solution to this problem is presented.

One possible explanation for the lower IgG readings by ELISA is that IgG, like many proteins, may adhere readily to utensil surfaces and thus can be lost with serial dilutions of milk that are required to perform ELISA. Tween, an efficient non-ionic detergent, was added in both sample and wash buffers. The inclusion of Tween in ELISA buffers for improving assay precision is well recognized since it prevents irreversible hydrophobic interactions of proteins to polystyrene plates (Bullock & Walls, 1977). IgG detection was further reduced when Tween was absent from the assay buffer, thus showing the importance of the solubilizing properties of Tween in providing better access of the IgG to the coating antibody.

An alternative explanation for the relatively low ELISA estimates of IgG in milk concerns the presence of fat and other proteins, specifically casein, which may interfere with the binding efficiency of the antibody with coating antigen. Raw milk samples were defatted by both self-creaming and centrifugation and casein was also removed by isoelectric precipitation from the defatted samples producing whey. Casein, although a non-reactive protein, has been shown to be a very effective blocking agent and interference factor in solid phase ELISA for human IgG (Kenna *et al.*, 1985). The results of the present study showed that the suppressing factor in milk associated with low ELISA IgG measurement was not specific to milk fat, casein or other components present in the dialysable fractions. Further studies are required to identify specifically the interference factor(s) in milk which preclude accurate measurement of IgG levels.

In light of the fact that we could not eliminate the factors associated with the milk matrix that interfered with IgG quantification by prior selective sample extraction, the

commercial IgG alone could not be used as an appropriate standard for determining unknown IgG levels in bovine milk samples. Adding commercial IgG to UHT milk was examined as a possible calibration curve, since the UHT process is often considered to be severe enough to destroy all IgG, yet the interference factor attributed to the milk matrix would also be present. Unfortunately, UHT milk was found to contain detectable IgG by both ELISA and RID, and therefore an alternative approach was developed. Important to the successful development of the ELISA test for bovine IgG in milk was the parallel standard curves between commercial standard IgG and milk spiked with the same concentration of IgG. To make the ELISA assay more accurate with bovine milk samples, the IgG content in milk was first determined for a reference milk standard by RID. Once the IgG content of the raw milk sample was accurately determined by RID, it was used as the reference standard for the calibration curve. Thus both requirements, namely the accurately known amount of IgG and the presence of the matrix interference factor were satisfied. A highly significant relationship was obtained for milk IgG content of samples determined using as the standard either the reference milk IgG standard determined by RID and assayed in serial dilutions by ELISA or commercial IgG standard. The consistency of the relationship was very important for the accurate quantification of bovine milk IgG and allowed for correction of unknown milk samples for IgG content. Other workers measuring secretory IgA in human milk (Akerlund *et al.*, 1977) and milk IgG to specific microbial antigens (Loeffler & Norcross, 1985; Smith *et al.*, 1989) by ELISA have not reported the apparent discrepancy between ELISA and RID values in milk samples.

Immunoglobulins are not as heat-resistant as casein (Wills *et al.*, 1982) and their relative degradation when exposed to various thermal processes is reflected by the different levels measured in raw, pasteurized and sterilized milks. Other investigators have failed to detect significant IgG in commercial skim milk powder (Kobayashi *et al.*, 1991). Using the ELISA technique reported herein, we observed that undenatured IgG was present in milks following HTST pasteurization and spray drying procedures, but not in UHT processed or canned milks. Moreover, the different thermal processing procedures used by individual dairy processors may contribute to variable levels of IgG.

In summary, the sandwich ELISA test described here, which involved correction for interference of the milk matrix, is more sensitive than chromatographic and electrophoretic methods and agrees with literature values (Larson, 1957; Kobayashi *et al.*, 1991). Sample preparation protocols are simple and it is easier to perform and quicker than the alternative immunodiffusion method which requires considerably more antisera for analysis.

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