Tanaka *et al. International Breastfeeding Journal* (2024) 19:74
https://doi.org/10.1186/s13006-024-00680-7

International Breastfeeding Journal

METHODOLOGY Open Access

New latex agglutination assay for the determination of lactoferrin in human milk

Miori Tanaka[1*], Midori Date[1] and Katsumi Mizuno[1,2]

Abstract

Background Lactoferrin (LF) in human milk has various biological properties and contributes to the prevention of preterm birth complications. Enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used methods to measure LF in human milk, but this method is time-consuming and laborious. In Japanese human milk banks, the concentration of LF in donor human milk (DHM) is measured routinely. Here, we reported a rapid, simple, and accurate method for determining LF in human milk using a new reagent based on a latex agglutination assay.

Methods We obtained 208 human milk pools from 148 mothers, and samples were collected before and after Holder pasteurization. Milk samples were diluted 100- or 200-fold and LF concentrations were measured by a latex agglutination assay using an automated analyzer. The reagent was validated in terms of repeatability, linearity, detection limit, recovery, and comparison with ELISA.

Results The coefficient of variation (CV) for intra-assay precision ranged from 0.6 to 5.0% in human milk with high, medium, and low LF concentrations. The linearity was also tested by serial sample dilution and was confirmed up to 16 µg/mL with a detection limit of 0.2 µg/mL. The recovery rates in a spiked recovery test were ranged from 90 to 120% at high, medium, and low concentrations of LF. Furthermore, a strong correlation was observed between LF levels determined by the latex agglutination assay and ELISA ($r = 0.978$, $p < 0.001$, $n = 255$). The regression equation was $y = 0.991x + 0.545$ ($r^2 = 0.974$, $p < 0.001$). Compared with ELISA, the latex agglutination assay reduces the measurement time by 160 min and the cost by 55%.

Conclusions The latex agglutination assay used to determine LF in human milk is rapid, simple, and accurate enough to be used routinely. Its use may contribute to the quick and easy provision of appropriate DHM to preterm infants.

Keywords Human milk, Lactoferrin, Latex agglutination assay

*Correspondence:
Miori Tanaka
miori.tanaka@milkbank.or.jp
[1]The Nippon Foundation Human Milk Bank, Nihonbashi-koamicho Square Building 1F, 17-10 Nihonbashi-koamicho, Chuo-ku, Tokyo 103-0016, Japan
[2]Department of Pediatrics, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

Background

Lactoferrin (LF) is a glycoprotein member of the transferrin family with a molecular weight of approximately 80 kDa and 60% sequence homology with serum transferrin [1]. LF was first discovered and isolated from bovine milk in 1939 [2] and was later isolated from both human and bovine milk in 1960 [3–5]. In human milk, LF is the most abundant protein in the whey fraction [6]. The concentrations of LF in colostrum and mature milk are >5 g/L and 1–3 g/L, respectively [7, 8], comprising 15–20% of their total protein content. Compared with human milk, LF levels in bovine milk are relatively low: 1.5 mg/L in colostrum and 0.5 mg/L in mature milk [9]. LF has a high affinity for free iron, limiting the availability of iron necessary for bacterial growth [1]. A previous in vitro study reported that human LF was more effective in inhibiting bacterial growth than bovine LF [10]. Also, LF exhibits antiviral, antifungal, anti-inflammatory, and immunomodulatory activities [1, 11–13].

Clinical trials have demonstrated the preventive effects of LF against sepsis and diarrhea in preterm infants [14, 15]. A systematic review and meta-analysis that included 9 randomized controlled trials showed that prophylactic LF significantly reduced the incidence of necrotizing enterocolitis (NEC) and late-onset sepsis [16]. In addition, preterm infants fed human milk with high LF levels had larger total brain and gray matter volumes than preterm infants exposed to low LF [17]. Thus, LF intake is related to both the prevention of complications and cognitive development in preterm infants. When there is an insufficient volume of the maternal milk for preterm infants, donor human milk (DHM) from human milk banks is recommended for bridging or replacement feeding [18, 19]. The availability of DHM in hospitals has shown to be associated with a reduced incidence of NEC [20, 21], which may be due to various bioactive substances in DHM, including LF. Holder pasteurization, the method used most frequently in human milk banks worldwide, greatly reduces bioactive components in human milk [22]. In addition, milk composition is highly variable and influenced by maternal and infant factors [23]. Therefore, Japanese human milk banks measure the nutrient composition (macronutrients, micronutrients, secretory immunoglobulin A, and LF) of DHM.

Currently, several methods to detect LF have been developed, including radial immunodiffusion, enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography, sensors, microarrays, and protein chips, each with advantages and disadvantages [24–26]. ELISA is one of the most commonly used methods for measuring LF in human milk and is used in Japanese human milk banks. Although ELISA is an accurate immunological analysis, this method is time-consuming and laborious, and the reagent kits are relatively expensive, making it difficult to use for routine measurements. Therefore, it is necessary to establish a rapid, accurate, simple, and less expensive method for LF detection.

Latex agglutination assay is based on the agglutination reaction that occurs when antibodies or antigens coated on latex beads bind to antigens or antibodies, and has been widely used to detect specific viruses and serum proteins in biological samples [27, 28]. Validation of a latex agglutination test for measuring bovine LF in dairy products has been reported [29]; however, until recently, no reagents have been available to assay LF in human milk. In this study, we developed and validated a new latex reagent for LF measurement in human milk in terms of repeatability, linearity, limit of detection, recovery, and comparison with ELISA.

Methods

Milk samples

This study was conducted at The Nippon Foundation Human Milk Bank (TNFHMB) and approved by the Showa University Research Ethics Review Board (approval number: 2714). Human milk samples were collected from donors who had completed a medical interview and blood screening test. Milk was obtained by hand or pump expression. After expression, milk in clean bags was frozen and stored in a freezer at the donor's home. Refrigerated transport ensures a cold transport chain for milk from the donor's home to TNFHMB or the Japan Human Milk Bank Association (JHMBA). On arrival at TNFHMB or JHMBA, milk was stored at -30℃ until pasteurization. Frozen milk was thawed in a refrigerator overnight and then pasteurized within 24 h. In preparation for pasteurization, thawed milk was pooled with other milk from the same donor to reduce nutrient variability. A total of 208 human milk pools were obtained from 148 donors, and samples were collected after pasteurization. For 58 of these samples, we also collected samples from each pool before pasteurization. Holder pasteurization (62.5℃, 30 min) was performed with a Sterifeed S90 pasteurizer (MediCare Colgate, Kentisbeare, UK), Barkey clinitherm pasteur 10/80 (Barkey, Leopoldshöhe, Germany), or Racoon dry pasteurizer HMP-4 (Mita Rika Kogyo, Osaka, Japan). Milk samples before and after pasteurization were stored at -80℃ until the respective tests (Fig. 1). All donors provided written consent for the use of their human milk for research purposes.

Reagents

Latex Test BL Lactoferrin (Reagents 1 and 2), lactoferrin calibrator, lactoferrin control, and diluted solution were purchased from Biolinks (Kanagawa, Japan).

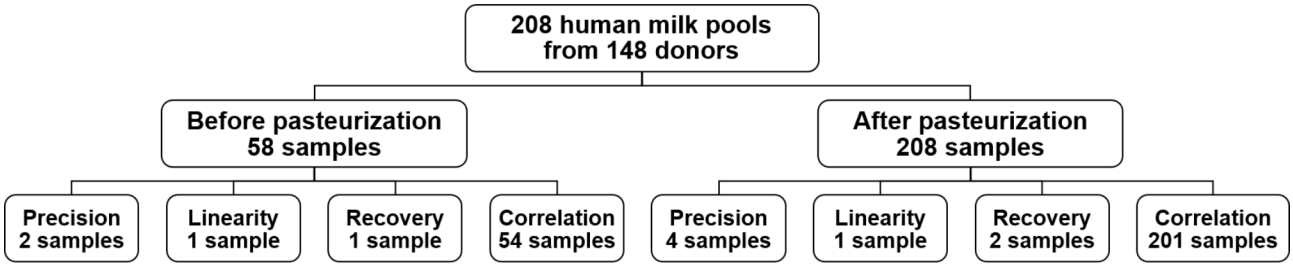

Fig. 1 Sample preparation flow chart

Latex agglutination assay procedure

LF levels were determined by a latex agglutination assay according to the reagent manufacturer's instructions. Milk samples were diluted 100-fold with a diluted solution, and samples with values exceeding 16 μg/mL were diluted 200-fold. The measurements were automatically performed using a CA-270 Clinical Chemistry Analyzer (Furuno Electric, Hyogo, Japan) as follows: 4 μL of diluted milk sample and 100 μL of buffer solution (Reagent 1) were pipetted into a cuvette and after approximately 5 min, 100 μL of polystyrene latex particles coated with anti-human LF mouse monoclonal antibody (Reagent 2) was added. The absorbance was then measured at 600 nm approximately 30 s and 5 min after adding Reagent 2, and the changes in absorbance due to agglutination were calculated. The concentration of LF in the milk samples was quantified by comparison with a spline calibration curve obtained from a lactoferrin calibrator. We checked that the concentrations of lactoferrin control were within range before measuring human milk samples.

Precision

To evaluate precision, a repeatability (intra-assay) study was carried out within a day. 10 replicate measurements were performed using 6 different human milk samples with high, medium, and low LF concentrations.

Linearity

We diluted 2 human milk samples with high LF concentrations 100-fold, and the linearity of the method was studied by analyzing 5 serial dilutions of these diluted samples.

Determination of the detection limit

We made 5 replicate measurements using LF isolated from human milk (L0520; Sigma-Aldrich, St Louis, MO, USA) with 3 different concentrations. The minimum concentration at which the mean − 2.6 SD of each concentration did not overlap with the mean + 2.6 SD of the blank (diluted solution) was considered as the detection limit [30].

Recovery test

We supplemented 3 human milk samples with a lactoferrin calibrator (final concentration: 2.1 μg/mL), and LF levels were measured. The percentage of recovery was calculated as follows:

Recovery = (C1-C2)/C3 × 100%

C1: Sample concentration after adding calibrator.

C2: Sample concentration before adding calibrator.

C3: Final concentration of adding calibrator.

Comparison with ELISA

LF levels in 255 human milk samples determined by a latex agglutination assay were compared with those measured by ELISA. For ELISA, milk samples were centrifuged at 3000 × *g* for 15 min at 4℃ to remove the fat layer, enabling the lower aqueous layer to be collected for analysis. To prepare a 1/50,000 sample dilution, 5 μL of sample was transferred into 495 μL of diluent solution. Next, 2 μL of the 1/100 sample was transferred into 998 μL of diluent solution. The concentration of LF was measured using a Human Lactoferrin ELISA Kit (E-80LF; Immunology Consultants Laboratory, Portland, OR, USA), following the manufacturer's instructions. The detection range and sensitivity of the kit were 3.125–100 ng/mL and 0.725 ng/mL, respectively.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 9 software package (GraphPad Software, La Jolla, CA, USA). All continuous variables were tested for normality by a D'Agostino–Pearson test. The relationship between the latex agglutination assay and ELISA results was determined using Spearman's rank correlation coefficient because the data exhibited a nonparametric distribution. Simple linear regression analysis was performed to examine the relationship between 2 variables. Differences were considered statistically significant when $p<0.05$.

Results

Precision

We estimated the intra-assay precision from 10 replicate analyses for LF in 6 human milk samples. The mean,

Table 1 Intra-assay precision of the latex agglutination assay ($n=10$)

Sample	Mean (μg/mL)	SD	CV (%)
High 1	15.1	0.09	0.6
High 2	14.6	0.26	1.8
Medium 1	5.0	0.08	1.6
Medium 2	4.9	0.11	2.2
Low 1	1.4	0.05	3.7
Low 2	1.2	0.06	5.0

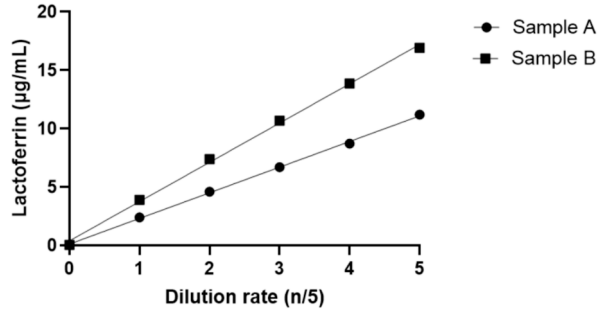

Fig. 2 Linearity test for the latex agglutination assay. LF levels were measured using 5 serial dilutions of 2 human milk sample

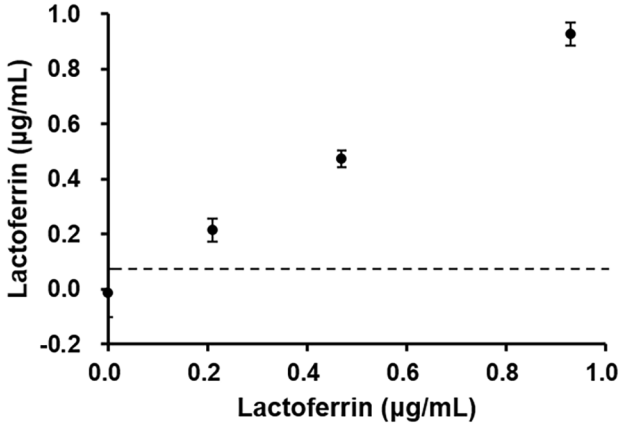

Fig. 3 Detection limit analysis for a latex agglutination assay. 5 replicate measurements were performed using LF authentic samples with 3 different concentrations. The points and error bars indicate the means ± 2.6 SDs. The dotted line indicates the mean + 2.6 SD of the blank

standard deviation (SD), and coefficient of variation (CV) for each sample are shown in Table 1. The CVs ranged from 0.6 to 5.0%, which is considered good precision for a routine method.

Linearity

To demonstrate the linearity, a regression line was prepared using 2 human milk samples in stepwise dilutions ranging from 11.2 to 2.4 μg/mL (sample A) and 16.9 to 3.9 μg/mL (sample B) (Fig. 2). A regression line was an excellent fit to the points in both samples (sample A: $r^2=0.999$, sample B: $r^2=0.998$), confirming the linearity of the assay within the range tested.

Table 2 Recovery test for the latex agglutination assay

Sample	C1 (μg/mL)	C2 (μg/mL)	C3 (μg/mL)	Recovery (%)
High	17.0	14.5	2.1	119.3
Medium	7.1	5.0	2.1	100.7
Low	2.7	0.8	2.1	91.0

Recovery = (C1-C2)/C3 × 100%

C1: Sample concentration after adding calibrator.

C2: Sample concentration before adding calibrator.

C3: Final concentration of adding calibrator.

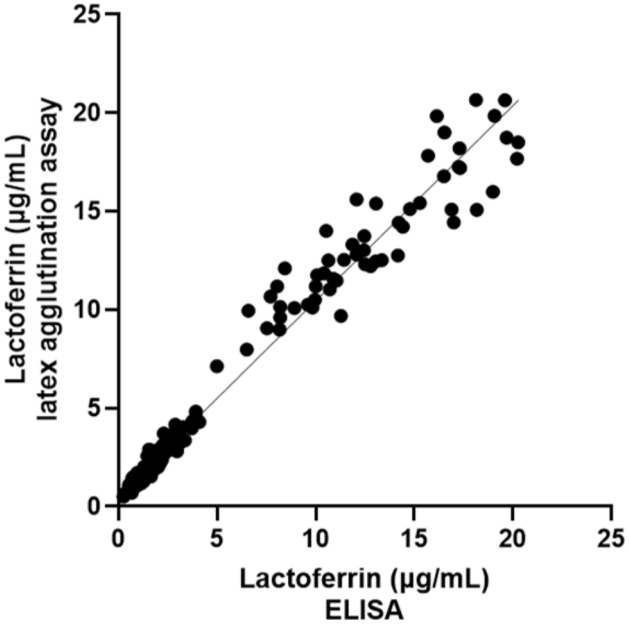

Fig. 4 Correlation between the latex agglutination assay and ELISA for LF determination in human milk. LF levels in 255 human milk samples were measured by the latex agglutination assay and ELISA ($y=0.991x+0.545$, $r=0.978$, $p<0.001$)

Determination of the detection limit

In the detection limit assay, the mean+2.6 SD of the blank was 0.07, and the mean – 2.6 SD of the sample containing 0.2 μg/mL LF was 0.17 (Fig. 3). The detection limit of the assay was therefore set at 0.2 μg/mL.

Recovery test

We calculated the percentage of recovery by measuring LF concentrations in 3 human milk samples before and after adding a lactoferrin calibrator. As shown in Table 2, the recovery rates were between 90% and 120%.

Comparison with ELISA

We analyzed 255 human milk samples by a latex agglutination assay (y) and ELISA (x). As shown in Fig. 4, a strong correlation was observed between LF levels determined by the latex agglutination assay and

ELISA (r=0.978, p<0.001). The regression equation was y=0.991x+0.545 (r^2=0.974, p<0.001).

Discussion

Latex agglutination assay was first used for the detection of rheumatoid factor in 1956 [31] and has since been developed to diagnose specific infectious and autoimmune diseases. Latex agglutination test has become increasingly automated, and antigen detection using monoclonal antibodies is particularly specific and straightforward. Polystyrene latex beads are commonly used because of their strong hydrophobicity, which makes them ideal for the passive adsorption of substances [28]. In the present study, a new latex reagent using these techniques was validated to develop a rapid, accurate, simple, and less expensive alternative method for determining LF levels in human milk.

The validation study demonstrated that the precision was good for a routine method as the CVs of the repeatability assay ranged from 0.6 to 5.0% in human milk with high, medium, and low LF concentrations. Linearity was confirmed up to 16 μg/mL, and the detection limit was 0.2 μg/mL, so the detection range for this reagent was determined to be 0.2–16 μg/mL. We also performed a recovery test to determine the non-specific agglutination effect of the matrices. There was a slight inhibitory effect of the matrices on agglutination in human milk, irrespective of LF concentration, as the recovery rates were 90–120% at high, medium, and low concentrations of LF. ELISA is more sensitive than the latex agglutination assay, with a detection range of approximately 0.5–100 ng/mL, depending on the kit. LF concentrations in human milk before and after pasteurization were reported to be approximately 2–3 mg/mL and 0.03–0.3 mg/mL, respectively [32, 33]. Thus, it is noteworthy that combining the low detection limit of ELISA and high dilution factor may eliminate the matrix effect more effectively than the latex agglutination assay. Finally, correlation analysis between ELISA and latex agglutination assay revealed a strong correlation, with a correlation coefficient (r) of 0.978 and a regression equation of y=0.991x+0.545. These results confirm the precision, linearity, and accuracy of the latex agglutination assay to quantify LF in human milk before and after pasteurization.

LF concentrations in human milk are influenced by various maternal and infant-related factors, including lactation stage (colostrum, transitional, and mature), geographical location, ethnicity, and birth weight [8, 9, 23, 34]. We previously reported that a negative correlation was found between postpartum week and LF levels of DHM in Japan [35]. Human milk banks provide several types of DHM obtained from donors at different gestational and postpartum weeks, with varying LF concentrations. Additionally, previous studies have shown that Holder pasteurization process greatly reduces LF content in human milk [22, 36] and that dry pasteurization resulted in more than 20% higher reduction in LF than water pasteurization [37].

Table 3 Comparison between the latex agglutination assay and ELISA to determine LF in human milk

	Latex agglutination assay	ELISA
Detection range	0.2–16 μg/mL	0.5–100 ng/mL
Dilution factor	100 or 200	50,000
Measurement time per 40 samples	30 min	190 min
Measurement cost per 40 samples	¥36,000	¥80,000

LF plays an important role in preventing preterm birth complications and in developing cognitive function [14–17]. However, LF variability in DHM may represent a major challenge in preventing complications and ensuring adequate postnatal growth. Nutritional analysis of DHM would help to assign the most appropriate DHM to each preterm infant, such as by assigning DHM with higher LF levels to preterm infants with a shorter gestational age and lower birth weight. In addition, because pooling DHM from multiple donors effectively eliminates variability in DHM composition [38], pooling based on LF measurements may better optimize LF content in DHM. Determining LF levels by the latex agglutination assay using an automated analyzer does not involve complicated steps, can control human error, and reduces the measurement time (latex: 30 min vs. ELISA: 190 min per 40 samples), leading to rapid clinical decisions on the selection of DHM provided to preterm infants in human milk banks. The new latex agglutination reagent can be easily adapted to automated analyzers available in most hospital laboratories. This makes it ready for use in neonatal intensive care units, where DHM and human milk are used to feed newborn infants. Furthermore, in our laboratory, this method reduced the cost of LF determination by 55% (latex: ¥36,000 vs. ELISA: ¥80,000 per 40 samples). For these reasons, this rapid and simple technique is considered suitable for routine measurement of LF in human milk. Table 3 compares the latex agglutination assay and ELISA for LF determination in human milk.

Conclusions

In conclusion, the latex agglutination assay is a rapid and simple method for determining LF in human milk and correlates well with ELISA method. This assay is expected to facilitate the timely and convenient provision of DHM appropriate to the background of each preterm infant.

Abbreviations

DHM Donor human milk
ELISA Enzyme-linked immunosorbent assay

JHMBA	Japan Human Milk Bank Association
LF	Lactoferrin
NEC	Necrotizing enterocolitis
TNFHMB	The Nippon Foundation Human Milk Bank

Acknowledgements
The authors thank all of the mothers who participated in this study for providing human milk samples.

Author contributions
M.T. designed the study; M.T. and M.D. collected the data; M.T. and M.D. analyzed the data; M.T. wrote the manuscript; K.M. supervised the study. All authors read and approved the final manuscript.

Funding
Not applicable.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
was obtained from Showa University Research Ethics Review Board (approval number: 2714; June 21, 2021). All donors provided written consent for the use of their human milk for research purposes.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 26 April 2024 / Accepted: 27 October 2024
Published online: 31 October 2024

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